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German Translation

Clinical Aspects and Treatment of Acetic Acid Intoxication

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The relatively frequent occurrence (1,3,12) prior to World War I, of often fatal cases of poisoning after peroral intake of so-called vinegar essence (about 80% acetic acid) has been reported, mainly around the turn of the century, in several case reports (4,6,10,22,33/34,35,44,48,50/51,60,62, 64). Later, this type of intoxication appears to have become less frequent, as pointed out by Friedrick-Breuniger (13), Kogler (26/27) and Siebert (54), so that relatively few authors (16-18,24,26/27,40,42,53,55,58/59,61) have been concerned with it. This fact explains why acetic acid poisoning is often not mentioned at all in toxicology textbooks or reviews (9,15,30,43, 45,56,65) or only briefly described (7,14,23,38). In this respect, there are only a few exceptions (11,19/20,25,32,39,52), which, however, are not quite satisfactory.

For this reason, we have already briefly reported elsewhere (28), on hand of 3 personal observations, the main clinical symptons of this disease. It was pointed out that, in severe cases, apart from local corrosion symptons, a more or less marked acidosis and/or hemolysis and shock are observed, during the course of which an acute kidney failure takes place. Other problems arising in this connection, e.g. the development of a coagulation disturbance, could only be touched upon briefly at that time (28), due to the lack of sufficient experience.

A further case of severe acetic acid intoxication, observed in the meantime, prompts us to describe this disease more extensively. This appears to be all the more justified as the cause of contradictory information on the occurence of oxy- and methemoglobin (24-26,28,50,55,59) can possibly be clarified in this manner.

Survey of Observed Cases

The first two cases (case 1 and 2 of ref. 28) included patients in which, along with corrosion after-effects, an acute kidney failure played a predominant role in the clinical picture. Both cases came to our clinic only on the 3rd day after treatment, so that the initial acidosis could not be objectively detected at all, and hemolysis could only be partially detected.

Case 1: (Theodor G., Patient record no. 635/60) was a 37-year old man (alcoholic) who drank about 100 ml vinegar essence for the purpose of committing suicide, whereby part of this amount had probably been expectorated. He had collapsed a few minutes later, was then taken to an outside hospital and there had vomited a large amount of blood several times. The urine amount collected during the first 24 hours was only 350 ml, and the urine had a "red-black" color, but did not contain any erythrocytes, which was attributed to an intravasal hemolysis, although this fact was not determined more precisely. Serum bilirubin on the 2nd day amounted to only 1.22 mg%, of which 0.64 mg% was indirect bilirubin. Treatment at first included intravenous infusions of large amounts of $NaHCO_3$ and several blood transfusions, after which the blood pressure quickly became normal. During the first 48 hours, spasmodic attacks of pain occurred in the middle portion of the body. Starting on the afternoon of the second day, the patient became anuric, and for this reason was transferred to our clinic. By this time (3rd day) serum bilirubin was

already normal. Hemoglobin amounted to 90%, the alkali reserve was 63 vol.% CO_2, the pH of venous blood was 7.46. The clinical picture in the following weeks was dominated by the following 2 symptons: 1) a typical picture of severe acute kidney failure, and 2) bleeding from the gastro-intestinal duct, frequently of a high degree and lasting for weeks (tarry and bloody stools, occasional blood vomiting), which could be controlled only with great difficulty. Anuria lasted for a total of 14 days. This renal insufficiency with repeatedly threatening hyperkalemias and residual N increases could be overcome by 2 hemodialyses on the 6th and 11th day and by a peritoneal dialysis on the 18th day (for further details, see ref. 28). The urine contained at first up to 8% protein according to Esbach (1.2 protein/day maximum) and a much larger number of erythrocytes, leucocytes and granulated cylinders than normal. From the 22nd day on, a proteinuria could no longer be detected. The polyuric phase reached its peak at the end of the 4th week with a daily urine amount of 7.6 l and was complicated in its further course by a parotitis of short duration, an infection of the efferent urinary tract lasting several weeks and of genital organs, and by a severe thrombophlebitis on the left lower leg. By the end of the 7th week, the urinary sediment was negative, at the start of the 10th week no pathological findings were detected in the bladder by cystoscopy, indigocarmine excretion was normal on both sides, and the renal pelvis was normal on retrograde filling. During the 14th week, a renal clearance gave approximately normal values (C_{In}=120 ml/min, C_{PAH}=490 ml/min, FF = 24.5%, Tm_{PAH}=47 mg/min), and the kidneys concentrated up to a specific weight of 1.026 in the concentration (thirst) test. From the 5th week on, no tarry stools were observed, and starting with the 6th

week concealed (occult) blood was absent (benzidine test). An X-ray examination of the upper digestive tract 10 weeks after the intoxication did not disclose, surprisingly, any definite signs of strictures or other corrosion after effects. At that time, a fractional study of the gastric juice showed that it was only slightly subacid.

During the 13th week, a thorough examination revealed a greater damage of the liver parenchyma (enlargement by 2 fingerbreadths; positive (+) galactose test; bromsulphthalein test: dye retention after 15 min 54%, after 45 min 32%; electrophoretic diagram of the serum: total protein 7.6 g%, including 2.8 g% albumins, 0.2 g% α_1-globulins, 0.2 g% α_2-globulins, 0.8 g% β-globulins and 3.6 g% γ-globulins; positive (+) vitamin K test). During a control test 7 months after intoxication, the above findings showed a partial improvement (liver enlarged by one fingerbreadth; galactose test ++; bromsulphthalein test: 32% after 15 min; 12% after 45 min; electrophoretic diagram of the serum: total protein 6.7 g%, including 3.2 g% albumins, 0.2 g% α_1-globulins, 0.7 g% α_2-globulins, 1.1 g% β-globulins, and 1.5 g% γ-globulins).

Case 2 (Edith M., patient record no. 3387/60) was a 37-year old woman, who, during a schizophrenic attack reportedly drank about 200 ml vinegar essence, part of which was probably expectorated. After treatment in several other hospitals, where she received milk, burnt magnesia, etc. and had collapsed several times, she was brought to our clinic only on the 3rd day in view of tarry stools and an increasing oliguria and anuria. In spite of this fact, a very large amount of hemoglobin could still be detected in the urine by spectrophotometry, while the serum pH and the alkali reserve in venous blood essentially corresponded to the degree of uremia (for details, see ref. 28). The urine pH at first was 7.0, the titration acidity

was 2.5 meq/l. Daily checks showed a leucocyte number between 25,300 and 31,600/mm^3, the number of thrombocytes was normal during the entire observation period. A catheter specimen of urine contained a huge number of erythrocytes.

The lethal outcome of the process in the patient, who remained anuric, could no longer be stopped by a hemodialysis performed on the 6th day, which exerted a sufficient effect from a blood chemistry viewpoint. The patient died a few hours later from circulatory failure in an extremely severe psychotic state of agitation. Autopsy (see Note) revealed, along with a high-degree fulgurative hemorrhagic inflammation of the upper digestive tract extending up to the duodenum, a severe hemoglobinuric nephrosis with extensive necrobioses of the tubular epithelia. In addition, a histological study disclosed only a low-level, fine-drop fatty degeneration of liver cells.

The 3rd case (case 3 in ref. 28) was a case of vinegar essence intoxication treated at a relatively early time; its clinical picture, except for the intravasal hemolysis, was dominated by a high-level acidosis.

This case involved a 29-year old man (Franz Sch., patient record no. 3976/61), who drank about 75 ml vinegar essence for suicide purposes. He was admitted to our clinic 3 hours later, after receiving milk at home and repeatedly vomiting blood during his trip to the clinic. The initial blood pressure was 180/160 mm Hg, but dropped after about 1/2 hour to hardly measurable values, which barely responded to high Arterenol dosages. Serum hemoglobin was 1624 mg % (normal values: 3-5 mg%), whereby both absorption bands characteristic for oxyhemoglobin were detected by spectrophotometry. A coagulation analysis performed at the very beginning disclosed a lowering

Note: We wish to thank Prof. Dr. K. Lennert for his friendly transmission to us of the autopsy reports on cases 2,3 and 6.

of the prothrombin complex with considerable hypofibrinogenemia and thrombocytopenia (28). By that time, the pH in venous blood had dropped to 7.11, and the alkali reserve to 24 vol % CO_2. Both values dropped to 7.05 and 19 vol % in spite of intravenous injection of 50 g $NaHCO_3$ and peroral administration of milk, egg albumin and burnt magnesia, and the patient died 10 hours after poisoning with symptoms of a highly severe acidosis. Autopsy disclosed that corrosion after-effects in the intestinal tract extended up to the jejunum. In addition, a marked hemoglobinuric nephrosis as well as a general hemorrhagic diathesis were noted (for details, see ref. 28).

Two further cases (case 4 and 5) of vinegar essence intoxication, which also took place for suicide purposes, had a light course free of complications. These cases merely exhibited local corrosion after-effects, but no significant general symptoms, and particularly no clinically detectable signs pointing to at least a stronger hemodialysis.

Case 4 (Hans A., patient record no. 3229/61) was a 51-year old man with an endogenous depression, who was admitted to our clinic a few hours after drinking an unknown amount of vinegar essence. The initial blood pressure had dropped to 90/60 mm Hg, but rose rapidly to reach normal values after intravenous injection of $NaHCO_3$ and dried human serum. Superficial corrosion effects, which healed rapidly, could be detected in the mouth cavity, pharynx and, as far as could be seen, also in the esophagus. The maximum number of leucocytes was 20,300/mm^3. The temperature reached 37.7°C only on the 2nd day. No complications in the kidneys were noted.

Case 5 (Anna R., patient record no. 4045/60) was a 44-year old woman with an endogenous depression, who drank only a few gulps of vinegar essence, had received large amounts of milk immediately afterwards, and was brought

indirectly to our clinic 1/2 day later. No symptoms of collapse could be determined from the case history, and the corrosion after-effects in the throat were very slight. Leucocytes reached a maximum number of 13,500/mm^3, the temperature in the first few days was 37.9°C. Residual N and serum bilirubin remained normal, and no complications were noted.

The final case again involved a severe acetic acid intoxication, which, on the basis of previous experience, was treated in a particularly intensive manner already at an early stage. Acute renal insufficiency did not take place, in spite of an extremely severe hemolysis, but the patient died several days later from heart and circulatory failure.

The 6th case (Adalbert E., patient record no. 1349162) was a 40-year old man with a history of blindness acquired a long time ago, who drank 2 cups of vinegar essence for suicide purposes. An unknown amount of poison was expectorated and possibly also aspirated (breathed). The patient was brought just 2 hours later to the local Throat, Nose and Ear Clinic, where a tracheotomy was performed and at first 500 ml of a 5% $NaHCO_3$ solution was given by intravenous infusion. After another hour, the patient came to our clinic, where the disease initially followed the course illustrated in Fig. 1. This figure shows at the same time the most important findings observed in the first stage of the process. At first, the serum hemoglobin was 1900 mg%, the pH in arterial blood 7.30, and the urine pH 6.45. The initially considerably depressed respiration was followed, after a total intravenous injection of 85 g $NaHCO_3$, by an apnoic phase, which required a temporary artificial respiration from a Poliomat apparatus. Under copious intravenous infusion of levulose instead of $NaHCO_3$, the patient continuously passed urine, which was collected in advance with a permanent catheter. Following

a renewed depressed respiration and after an intravenous infusion of 7.5 g $NaHCO_3$, a transitory Cheyne-Stokes breathing set in, whereupon sodium bicarbonate was again replaced by intravenous levulose. A severe hemoptysis, occurring for the first time 13 hours after intoxication, led to a threatening drop in blood pressure, which could be rapidly restored, however, by volume filling and other therapeutic measures (see Fig. 1). By that time, the pH in arterial blood had become normal (7.40).

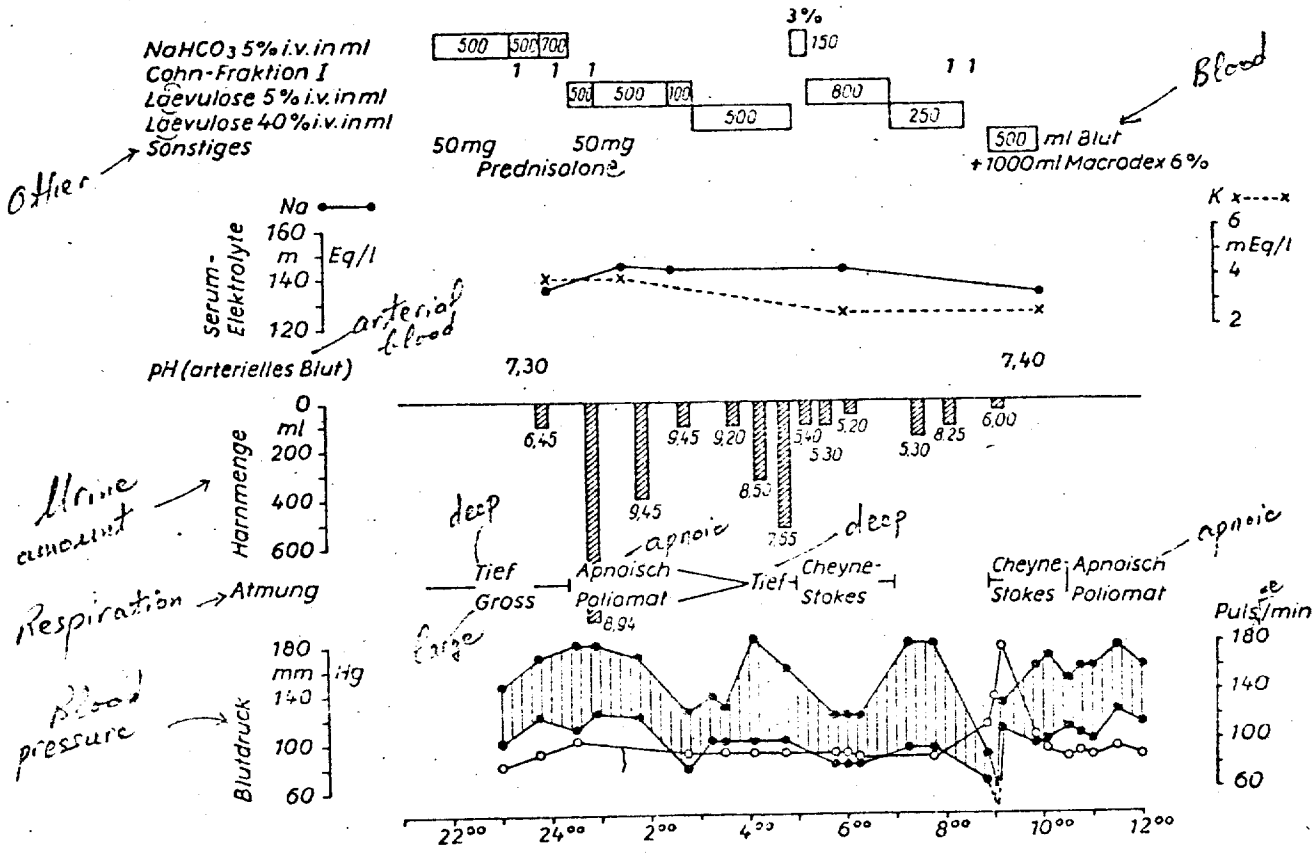

Fig. 1. Progress of a case of severe acetic acid poisoning in a 40-year old man during the first 17 hours. Figures under the columns, indicating urine amounts, represent the pH of urine. For further details see text.

The behavior of the prothrombin complex (prothrombin, factor V/VI and VII) and of the thromboelastogram (TEG) during this initial phase of the sickness is shown in Fig. 2. From this figure, we can see that the factors of the prothrombin complex dropped (decreased) to a lesser or greater extent during the first hours, whereby accelerator globulin reached an excessively low value (5%) about 5 hours after poisoning, and then rapidly returned to normal during the further course of the process. Particularly striking was the behavior (course) of the TEG, which was continuously recorded during the first phase until it became normal (Fig. 3). A significant lowering (decrease) of blood platelets could not be observed.

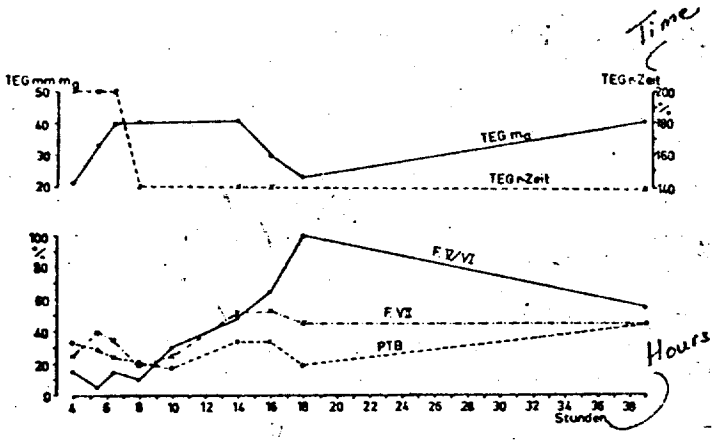

Fig. 2. Behavior of the thromboelastogram (TEG) and prothrombin complex (PTB = prothrombin, F. VII = Factor VII, F. V/VI = Factor V/VI) in a case of severe acetic acid poisoning during the initial phase of the sickness. For further details, see text.

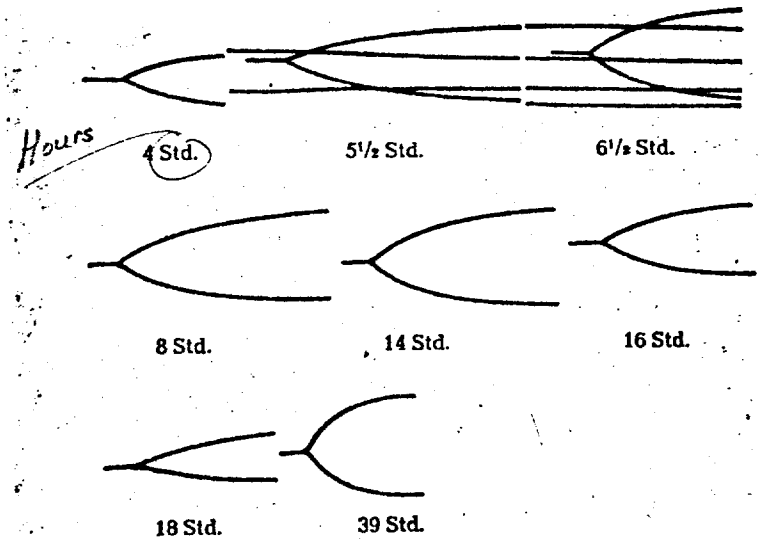

Fig. 3. Behavior of the thromboelastogram 4-39 hours after peroral intake of 2 cups of vinegar essence by a 40-year old man.

Figure 4 shows that urine contains only oxyhemoglobin 4 hours after poisoning, but no methemoglobin. From here on, a practically linear increase of the methemoglobin concentration is noted, with an opposite course of the oxyhemoglobin content of urine (Note: We wish to thank Doz. Dr. B. Hess at this point for the corresponding determinations by the cyan method).

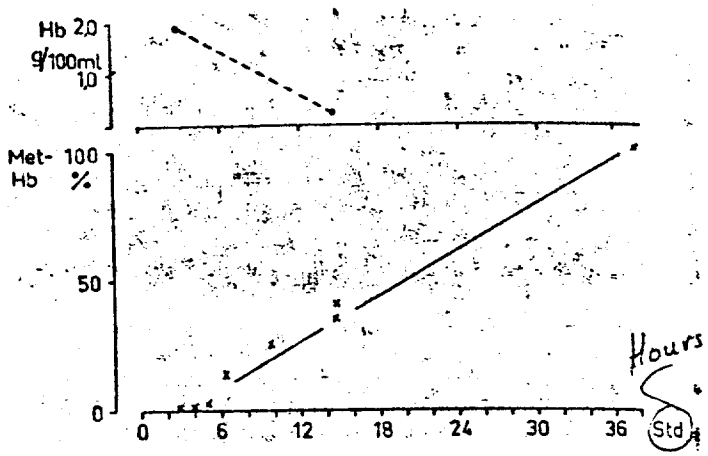

Fig. 4. Urine content of oxyhemoglobin (Hb) and methemoglobin (Met-Hb) in the first 1 1/2 day after peroral intake of 2 cups of vinegar essence by a 40-year old man. For further details, see test.

After the acute stage was overcome, circulation remained balanced in the following days, with a supply of liquids adjusted to fluid elimination. The temporarily reduced serum potassium content (see Fig. 1) could be restored again to its normal value by appropriate substitution. During the first 2 days, leucocytes reached a maximum number of 33,000/mm^3, the rectal temperature fluctuated in the first 4 days around 38°C. The serum bilirubin increased on the 2nd day to 2.9 mg%. The enzyme content of the serum exhibited the following values during the same time: LDH 1095 I.U., GOT = 300 I.U., GPT = 219 I.U., alkaline phosphatase = 8.5 I.U. Lactate dehydrogenase (LDH) dropped after 2 more days down to 758 I.U. Residual N remained normal during the entire period. After 3 days, the urine contained no more protein after an initial light proteinuria, but did contain occasionally granulated cylinders and isolated erythrocytes.

An X-ray examination of the thorax, performed 12 hours before death, disclosed partially confluent bronchopneumonic foci in both lower areas, as well as in the left midzone. In spite of intensive antibiotic treatment with chloramphenicol carried out from the very beginning, hyperpyretic temperatures appeared suddenly at this time, which could no longer be controlled therapeutically, so that the patient died from heart and circulatory failure on the 5th day after poisoning.

Autopsy disclosed high-grade corrosive burns of the mucous membrane extending up to the antrum ventriculi, as well as a severe pseudomembranous necrotizing inflammation of the larynx, the trachea and the bronchi on both sides, further extensive pancreas fatty tissue necroses, and a strong, primarily acinocentral, but at some points also diffuse, medium-to large-drop fatty degeneration of liver cells with small-foci liver cell necroses.

In addition, multiple partly blotched and partially punctiform hemorrhages were noted on both sides of the pleura visceralis, as well as a hemorrhagic pleural effusion (300 ml) on the right side and blotched subendocardial hemorrhages in the outflow (discharge) circuit of the right ventricle. A histological examination of the kidneys showed a low-level hemoglobinuric nephrosis and a diffuse, fine-drop fatty degeneration of the renal tubule epithelia.

Discussion

From the description of these 6 patients suffering from vinegar essence intoxication, observed at our clinic in the past 3 years, we can obtain the entire symptomatological scale of this syndrome. The light cases (cases 4 and 5) are dominated by local corrosive after-effects, without the appearance of any significant (or none at all) intravasal hemolysis. The corresponding mucous membrane lesions heal relatively fast and leave behind no permanent residues, as far as could be judged from the available observations. In severe cases of poisoning, a more or less marked acidosis (cases 3 and 6) is additionally noted, on the one hand, and occasionally a high-level hemolysis (cases 2,3 and 6) on the other hand. The latter has already been reported by different authors (18,22,25,26,33/34,40,42,50,53,55,58,59,61,62), whereas acidosis was mentioned in only isolated clinical studies (19,58). In addition, a more or less high-level shock (cases 1-3) is observed in the initial stage of the process, in agreement with various authors (6,22,34,40, 48,50,64). The further clinical picture is determined, as long as it not possible to therapeutically influence the acidotic metabolic condition and/or the shock at the right time, by the typical picture of acute kidney

failure (case 1 and 2); the problem posed by the latter has already been discussed elsewhere (28).

The pathogenesis of shock, acidosis and hemolysis after peroral intake of vinegar essence is no doubt a very complex problem, which will be discussed here only in broad terms, since its further clarification will be attempted by means of experimental animal studies (8). On the basis of studies carried out by Schibkow (52), we can assume that the direct corrosive action of vinegar essence on the mucous membranes of the oral cavity and of the upper intestinal tract can already cause a drop of the blood pressure, which is further reinforced by the vomiting of blood (hematemesis) that occasionally takes place very early, as was already noted by Gerhartz (16), Hitzig (22), Karber (24), Romeik (48) and Schaffer (50). At least in some cases, these factors may not be sufficient, however, to effectively determine the further development of the process. The most important pathogenetic factor, then, is the more or less clearly expressed acidosis, which can conceivably lead in different ways to an acute kidney failure (Fig. 5). One such possibility is the direct inducement or even aggravation of a shock with the consecutive development of shock kidneys. Since, according to animal experimental studies of Meesmann et al. (37), however, acidification alone, i.e. without any significant and simultaneous hemodynamic reactions (repercussions), can also result in a failure of the renal function, such a mechanism is also debatable when anuria and/or oliguria takes place. Finally, it is possible that a severe acidosis together with an intravasal hemolysis may lead to the appearance of chromoprotein kidneys. According to the experimental studies of Bing (2) and others (for further references, see 47), the simultaneous formation of methemoglobin appears indeed to be a prerequisite for the

effectiveness of such a pathogenetic chain, and it is then possible that intravasal hemolysis combined with a shock can give rise in this manner to the formation of a hemolytic kidney. This mechanism, which we have specifically pointed out recently (28), shows that the combination of the different factors pointed out here is probably the rule, and that a demarcation of individual partial components in an isolated case is probably impossible.

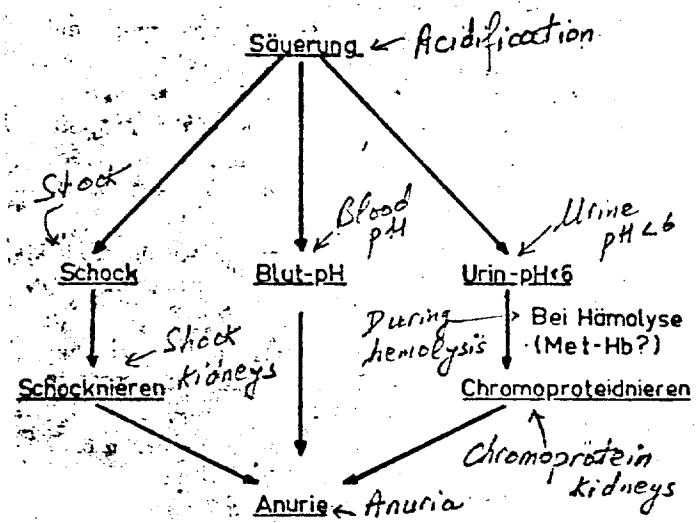

Fig. 5. Schematic diagram of the possible pathogenetic pathways along which acidoses can lead to acute kidney failure during acetic acid poisoning. For further details, see text.

The acidosis occurring in the initial stage of severe acetic acid poisoning probably also plays an important role in the development (formation) of hemolysis. In this connection, however, the lipophil character of acetic acid must also be taken into account as a causal factor, although its contribution to the pathogenesis of the hemolytic syndrome cannot be defined more precisely on the basis of the human experience reported here. In the final analysis, the same applies also to the cause of acidosis, whereupon this subject can be examined in greater detail during the course of experimental studies.

Our recent findings (28) showing that oxyhemoglobin, and not methemoglobin, is formed in the course of the hemolytic process during acetic acid poisoning make it necessary, in view of the results now available, to introduce a correction or, better stated, a supplementary remark. This statement, which appears to contradict the data reported by Karber (24), Kogler (26) and Strzyzowski and Nicod (59), is based on the spectrophotometric studies performed on our case 3 and resulted, as the results obtained with case 6 show (Fig. 4), at that time apparently from the fact that in case 3 only the initial stage of the process was investigated. It can be seen namely from Fig. 4 that the oxyhemoglobin actually present alone in the initial phase of the intoxication is converted more and more into methemoglobin during the further course of the process, so that the urine contains practically only methemoglobin about 14 hours after the onset on sickness. In this manner, the pertinent contradictions found in the literature can probably be explained: namely, in those cases where only oxyhemoglobin was found (20,50,55) apparently only the early stages of intoxication were studied, whereas in those cases where methemoglobin was detected (24,26,59) apparently the later phases were primarily investigated. Thus, it appears certain in any case that vinegar essence is not a primary methemoglobin former, but that this oxidation product of hemoglobin appears only gradually during acetic acid poisoning as a result of certain biological reactions.

Further, of special interest during severe acetic acid poisoning is the general hemorrhagic diathesis observed, which we have already briefly pointed out elsewhere (28) on hand of case 3.

This case disclosed, immediately prior to the onset of the blood pressure drop and in addition to the decrease of the prothrombin complex

(prothrombin, factors V/VI and VII), also a considerable hypofibrinogenemia with a thrombocytopenia. The behavior of blood platelets, thromboelastogram (Figs. 2 and 3) and prothrombin complex (Fig. 2) during the first 1 1/2 day after poisoning could now be followed continuously in a further case (case 6). In this case, during the first 10 hours after poisoning, factors of the prothrombin complex, and especially accelerator globulin (factor V/VI), showed a strong decrease, while thrombocytes remained approximately within a normal range. After 18 hours, factor V had at first become completely normal, but dropped again to 55% after a further 21 hours. A normalization of prothrombin and factor VII could not be observed during this time period. At the start of the intoxication, the TEG curves were greatly narrowed; a few hours later, the maximum amplitude of the TEG had reached normal values, but decreased again after 16 hours. The initially prolonged (longer) coagulation time was again finally normal already 4 hours after the start of treatment.

An interpretation of these findings is not possible yet. By appropriate buffering of our coagulation batches, it is out of the question that methodical errors based on acidification could have given rise to these values. An explanation of this fact is reported at the present time by Lausch et al. According to our experience, it can be stated already now that a considerable importance must be attributed to these coagulation changes in evaluating the severity of the intoxication.

The changes of the coagulation potential discussed here refer only to the initial phase of the process. The reductions (decreases) of the prothrombin complex occurring during later stages, such as those observed in case 1 and also suggested in case 6, are probably due to the more or

less severe liver injury taking place during the course of intoxication in case of a sufficiently long survival time. Such an additional organ lesion, which has been pointed out already by several authors (17-19,35,39-41), could be confirmed (when the results obtained by Gerlach et al. (18) are taken into account) on hand of pathological-anatomic data and appear likely already intra vitam (during life) in case 6 in view of the strikingly small LDH/GOT ratio. The clinical data in case 1 can also be interpreted in the same way, although here a pre-existing injury of the liver parenchyma must be assumed in view of the chronic drinking history of the patient. Nevertheless, a striking fact with this patient is that the size of the liver decreased during the many months of observation and that the liver function tests, and primarily the bromsulphthalein test, in part showed a definite tendency to improve. Further studies are necessary, however, to determine the cause responsible for the damage of liver cells in cases of acetic acid poisoning, where the patient survived for at least several days.

The question concerning an adequate treatment of acetic acid poisoning has so far been discussed by us (28) mainly in regard to the always present threat of an acute kidney failure. In connection with case 3, we have already pointed out that special therapeutic measures are necessary to control the high-level acidosis with all its consequences, which sometimes sets in rapidly in severe intoxication cases. The primary goal of such therapeutic measures will be to break, as early as possible, the vicious circle produced by shock, acidosis and hemolysis, and thus to avoid at least the development of a kidney failure. On the basis of the experience gained in the mean time, this could actually be achieved in the last case observed by us (Fig. 1). Starting from the ideas formulated during the above-mentioned

considerations (see Fig. 5), we believe, first of all, that the intravenous application of sodium bicarbonate in a maximum dosage is of decisive importance. The above-mentioned case teaches us, by the way, that therapy requires a continuous control of respiration. Therefore, a tracheotomy performed immediately, with the possibility of applying artificial respiration, represents a sine qua non condition. In case of breathing disorders (see Fig. 1), it is suggested that the above alkalization be temporarily discontinued. In this case, continuation of the infusion treatment with more or less high-percentage levulose is urgently recommended as long as the carefully supervised diuresis allows such a supply of liquid. In this manner, the critical first 24 hours could be overcome in the 6th case discussed here, in spite of the considerable hemoglobinuria present. Otherwise, the rules for the treatment of acute kidney failure are of course applicable. If a shock still cannot be avoided, in spite of all these measures, then one will have to keep in mind, among other things, that a lowered response of arterioles to angiotensin is found in a certain definite area during an acidotic metabolic condition, so that one must have recourse, if necessary, to an arterenol "Dauertropf" (continuous) infusion (36).

Thus, in general, the following therapeutic procedure is recommended for severe acetic acid poisoning, based on the experience available here:

1. Within the first 10-15 minutes: gastric lavage, followed by $Mg(OH)_2$ (38); otherwise and/or after:

2. Tracheotomy; simultaneously and as colaterally as possible:

3. Intravenous injection of 5% sodium bicarbonate (under continuous control of the depth of respiration); if necessary (cf. above):

4. Intravenous injection of 5% levulose (according to the degree of diuresis, which must be carefully checked). If the urine amount decreases, either again intravenous $NaHCO_3$ or 40% levulose (osmotic diuresis).

5. In case shock is already present, additionally the usual shock treatment (hypertensin or arterenol "Dauertropf" infusion, see above).

6. In case of reduced urination, the generally applicable rules for the treatment of acute kidney failure should be, followed.

Naturally, the further fate of the patient is determined, as case 6 shows, finally by the extent of corrosive burns. Further additional experience will be necessary in order to decide whether the always present threat of acute kidney failure during acetic acid poisoning can be avoided, at least in the majority of cases, by using the procedures described here.

Altogether, a knowledge of this intoxication and of its possible methods of treatment appears to be of increasing interest, since this type of poisoning apparently occurs more often than is indicated in the relatively scarce literature on this subject.

Summary

A total of 6 cases of intoxication following peroral intake of vinegar essence are reported; 2 of these cases were light and without any complications, whereas 6 cases had a a definitely severe course. Two of the latter cases exhibited the typical picture of an acute kidney failure, whereby one patient could be saved by means of extracorporeal hemodialysis and peritoneal dialysis. Another patient died within 10 hours in shock with a picture of very severe acidosis and hemolysis, whereas in the final case renal insufficiency could be prevented by adequate therapy, in spite of a

marked acidosis and hemolysis.

The clinical picture of the sickness is discussed in detail, with special attention devoted to the pathogenesis of acidosis, hemolysis, and eventually shock, and to their possible consequences (acute kidney failure). As a result of this study, a number of significant therapeutic measures for the treatment of the initial phase of acetic acid poisoning are suggested, which, as experience shows, can prevent the development of acute kidney failure in some cases.

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FORTBILDUNG

Klinik und Therapie der Essigsäure-Intoxikation

Aus der Medizinischen Universitätsklinik (Ludolf-Krehl-Klinik) Heidelberg (Direktor: Prof. Dr. K. Matthes)

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Das vor dem 1. Weltkrieg relativ häufige Vorkommen (1, 3, 12) von vielfach tödlichen Vergiftungen nach peroraler Einnahme von sogen. Essigessenz (= ca. 80%iger Essigsäure) hatte vor allem um die Jahrhundertwende in mehreren Fallberichten (4, 6, 10, 22, 33/34, 35, 44, 48, 50/51, 60, 62, 64) seinen Niederschlag gefunden. In der Folgezeit scheint diese Intoxikation, wie aus den Angaben von Friedrich-Breuninger (13), Kogler (26/27) und Siebert (54) hervorgeht, seltener geworden zu sein, so daß sich nur verhältnismäßig wenige Autoren (16—18, 24, 26/27, 40, 42, 53, 55, 58/59, 61) damit beschäftigt haben. Dies macht verständlich, warum die Essigsäure-Vergiftung in toxikologischen Lehrbüchern oder Übersichten oft entweder gar nicht (9, 15, 30, 43, 45, 56, 65) oder nur kurz (7, 14, 23, 38) beschrieben wird. In dieser Hinsicht bestehen nur wenige Ausnahmen (11, 19/20, 25, 32, 39, 52), die jedoch nicht ganz befriedigen.

Aus diesem Grunde hatten wir an Hand von 3 eigenen Beobachtungen bereits andernorts (28) über die wesentlichen klinischen Merkmale dieses Krankheitsbildes kurz berichtet. Es wurde hervorgehoben, daß es in schweren Fällen, abgesehen von den lokalen Verätzungserscheinungen, zu einer mehr oder weniger ausgeprägten Azidose und/oder Hämolyse sowie zu einem Schock komme, in deren Verlauf sich ein akutes Nierenversagen einstelle. Andere, in diesem Zusammenhang aufgetauchte Probleme, z. B. die Entwicklung einer Gerinnungsstörung, konnten seinerzeit (28) in Ermangelung ausreichender Erfahrungen nur gestreift werden.

Ein weiterer, inzwischen beobachteter Fall von schwerer Essigsäure-Intoxikation gibt Veranlassung, diese Erkrankung in größerem Umfange noch einmal zu erörtern. Dies erscheint um so gerechtfertigter, als hierbei auch die Ursache der widersprechenden Angaben über das Auftreten von Oxy- bzw. Met-Hämoglobin (24—26, 28, 50, 55, 59) geklärt werden konnte.

Übersicht über die beobachteten Fälle

Die beiden ersten Fälle (= Fall 1 und 2 von 28) waren Patienten, bei denen außer den lokalen Verätzungsfolgen ein akutes Nierenversagen im Vordergrund des klinischen Bildes stand. Beide Fälle kamen erst am 3. Tage anbehandelt in unsere Klinik, so daß sich eine initiale Azidose überhaupt nicht mehr, eine Hämolyse nur noch teilweise objektivieren ließen.

Fall 1: (Theodor G., Krankenbl.-Nr. 635/60) war ein 37jähr. Mann (Potator), der in suizidaler Absicht etwa 100 ml Essigessenz getrunken hatte, wovon allerdings ein Teil wahrscheinlich wieder hinausgewürgt worden war. Er war wenige Minuten später kollabiert, dann in ein auswärtiges Krankenhaus eingeliefert worden und hatte dort mehrmals im Schwall Blut erbrochen. Die Harnmenge der ersten 24 Stunden betrug nur 350 ml, wobei der Urin „rotschwarz" gewesen sei, jedoch keine Erythrozyten enthalten habe, was auf eine intravasale Hämolyse bezogen wurde, ohne daß dies genauer objektiviert worden wäre. Das Serum-Bilirubin betrug am 2. Tag lediglich 1,22 mg%, davon 0,64 mg% indirektes Bilirubin. — Die Behandlung erfolgte anfangs mit i.v. Infusionen großer Mengen von Na HCO_3 sowie mehreren Bluttransfusionen, worauf sich der Blutdruck rasch normalisierte. Während der ersten 48 Stunden bestanden anfallsweise auftretende, krampfartige Schmerzen im Mittelleib. Vom Nachmittag des 2. Tages an war der Patient anurisch, weswegen die Verlegung zu uns erfolgte. Das Serum-Bilirubin hatte sich zu dieser Zeit (= 3. Tag) bereits normalisiert. Das Hämoglobin betrug 90%, die Alkalireserve 63 Vol.% CO_2, das pH im venösen Blut 7,46. — Das klinische Bild war in den folgenden Wochen von zweierlei beherrscht: 1. Von dem typischen Bild eines schweren akuten Nierenversagens und 2. von wochenlang anhaltenden, vielfach hochgradigen Blutungen aus dem Magen-Darm-Kanal (Teer- und Blutstühle, gelegentlich Bluterbrechen), die nur mühsam beherrscht werden konnten. Die Anurie dauerte insgesamt 14 Tage. Diese renale Insuffizienz mit wiederholt bedrohlichen Hyperkaliämien bzw. Rest-N-Steigerungen konnte durch 2 Hämodialysen am 6. und 11. Tag sowie eine Peritonealdialyse am 18. Tag überwunden werden (weitere Einzelheiten:

28). Der Urin enthielt anfangs bis 8‰ Eiweiß nach Esbach (1,2 g Eiweiß/Tag maximal) sowie mehrmals vermehrt Erythrozyten, Leukozyten und granulierte Zylinder. Vom 22. Tage an war eine Proteinurie nicht mehr nachweisbar. Die polyurische Phase erreichte Ende der 4. Woche mit einer Tagesharnmenge von 7,6 l ihr Maximum und war im weiteren Verlauf kompliziert durch eine kurzdauernde Parotitis, eine mehrwöchige Infektion der ableitenden Harnwege sowie der Genitalorgane und eine schwere Thrombophlebitis am linken Unterschenkel. Ende der 7. Woche war das Harnsediment o. B., Anfang der 10. Woche die Blase zystoskopisch unauffällig, die Blauausscheidung beiderseits normal, Nierenbecken bei retrograder Füllung regelrecht. In der 14. Woche ergab eine renale Clearance annähernd normale Werte (C_{In} = 120 ml/min, C_{PAH} = 490 ml/min, FF = 24,5%, Tm_{PAH} = 47 mg/min), und die Nieren konzentrierten im Durstversuch bis zu einem spezifischen Gewicht von 1,026. — Von der 5. Woche an bestanden keine Teerstühle mehr, von der 6. Woche an war auch okkultes Blut (Benzidinprobe) nicht vorhanden. Bei einer röntgenologischen Untersuchung der oberen Verdauungswege 10 Wochen nach der Intoxikation fanden sich überraschenderweise keine sicheren Hinweise auf Strikturen oder sonstige Verätzungsfolgen. Der Magensaft war zu dieser Zeit bei fraktionierter Untersuchung nur leicht subazide.

In der 13. Woche sprach die weitere Durchuntersuchung für einen stärkeren Leberparenchymschaden (Vergrößerung um 2 QF; Galaktoseprobe −; Bromthalein-Test: Farbstoffretention nach 15 min 54%, nach 45 min 32%; Elektrophorese-Diagramm des Serums: Gesamtprotein 7,6 g%, davon 2,8 g% Albumine, 0,2 g% α_1-Globuline, 0,2 g% α_2-Globuline, 0,8 g% β-Globuline und 3,6 g% γ-Globuline; Vitamin-K-Test +). Bei einer Kontrolle 7 Monate post intoxicationem hatten sich die diesbezüglichen Befunde teilweise gebessert (Leber = 1 QF; Galaktoseprobe ++, Bromthalein-Test: 32%/15 min., 12%/45 min.; Elektrophorese-Diagramm des Serums: Gesamtprotein 6,7 g%, davon 3,2 g% Albumine, 0,2 g% α_1-Globuline, 0,7 g% α_2-Globuline, 1,1 g% β-Globuline und 1,5 g% γ-Globuline).

Fall 2: (Edith M., Krankenbl.-Nr. 3387/60) war eine 37jähr. Frau, die im Laufe eines schizophrenen Schubes angeblich etwa 200 ml Essigessenz getrunken hatte, wovon ein Teil vermutlich erbrochen wurde. Nach einer Behandlung in mehreren anderen Krankenhäusern, wo sie Milch, Magnesia usta usw. erhalten hatte und mehrmals kollabierte, war sie wegen Teerstühlen und einer zunehmenden Oligurie bzw. Anurie erst am 3. Tag in unsere Klinik gekommen. Trotzdem konnte im Urin spektrophotometrisch noch sehr reichlich Hämoglobin nachgewiesen werden, während das Serum-pH sowie die Alkalireserve im venösen Blut im wesentlichen dem Grad der Urämie entsprachen (Einzelheiten: 28). Das Urin-pH betrug zu Beginn 7,0, die Titrationsaziditat 2,5 mEq l. Die Leukozyten lagen bei täglicher Kontrolle zwischen 25 300 und 31 600/mm³, die Thrombozyten während der ganzen Beobachtungszeit im Normbereich. Der Katheter-Urin enthielt massenhaft Erythrozyten.

Der letale Verlauf* des Prozesses konnte bei der Patientin, die anurisch blieb, durch eine am 6. Tage vorgenommene Hämodialyse, die blutchemisch einen ausreichenden Effekt hatte, nicht mehr aufgehalten werden. Sie kam wenige Stunden später in einem schwersten psychotischen Erregungszustand am Kreislaufversagen ad exitum. Autoptisch* fanden sich neben einer hochgradigen, bis ins Duodenum reichenden verschorfenden hämorrhagischen Entzündung der oberen Verdauungswege eine schwere hämoglobinurische Nephrose mit ausgedehnten Nekrobiosen der Tubulusepithelien. Die histologische Untersuchung ergab darüber hinaus eine nur geringgradige feintropfige Verfettung der Leberzellen.

Der 3. Fall (= Fall 3 von 28) war eine relativ frühzeitig aufgenommene Essigessenz-Intoxikation, deren klinisches Bild, abgesehen von der intravasalen Hämolyse, von einer hochgradigen Azidose beherrscht war.

Es handelte sich um einen 29jähr. Mann (Franz Sch., 3976/61), der in suizidaler Absicht etwa 75 ml Essigessenz eingenommen hatte. Die Klinikaufnahme erfolgte 3 Stunden später, nachdem er zu Hause noch Milch erhalten und auf dem Transport wiederholt blutig erbrochen hatte. Der Blutdruck betrug zu Beginn 180/160 (!) mm Hg, sank jedoch nach etwa ½ Stunde auf fast nicht meßbare Werte ab, die auf hohe Arterenol-Dosen kaum ansprachen. Das Serum-Hämoglobin belief sich auf 1624 mg% (Normalwerte: 3—5 mg%), wobei spektrophotometrisch die beiden für Oxy-Hämoglobin charakteristischen Absorptionsbanden gefunden wurden. Eine ganz am Anfang vorgenommene Gerinnungsanalyse ergab eine Verminderung des Prothrombin-Komplexes mit erheblicher Hypofibrinogenämie und Thrombozytopenie (28). Das pH im venösen Blut war zu dieser Zeit auf 7,11, die Alkalireserve auf 24 Vol.% CO_2 abgefallen. Beide Werte sanken trotz 50 g Na HCO_3 i.v. und peroraler Gabe von Milch, Eiereiweiß und Magnesia usta auf 7,05 bzw. 19 Vol.% ab, und der Patient verstarb 10 Stunden post intoxicationem unter den Zeichen der schwersten Azidose. Autoptisch reichten die Verätzungsfolgen am Intestinaltrakt bis ins Jejunum. Darüber hinaus bestand eine ausgeprägte hämoglobinurische Nephrose sowie eine allgemeine hämorrhagische Diathese (Einzelheiten bei 28).

2 weitere Fälle (Fall 4 und 5) von Essigessenz-Intoxikation, die ebenfalls in suizidaler Absicht erfolgten, verliefen leicht und komplikationslos. Sie zeigten lediglich örtliche Verätzungsfolgen, jedoch keine wesentlichen Allgemeinerscheinungen, insbesondere keine klinisch faßbaren Hinweise auf eine zumindest stärkere Hämolyse.

Fall 4: (Hans A., Krankenbl.-Nr. 3229/61) war ein 51jähr. Mann mit einer endogenen Depression, der wenige Stunden nach der Einnahme einer unbekannten Menge von Essigessenz in unsere Klinik kam. Der Blutdruck war zu Beginn auf 90/60 mm Hg abgesunken, stieg aber unter i.v. Applikation von $NaHCO_3$ und Serumkonserven rasch auf normale Werte an. In Mundhöhle, Pharynx und, soweit einsehbar, auch Ösophagus waren oberflächliche Verätzungsfolgen nachweisbar, die rasch abheilten. Die Leukozyten betrugen maximal 20 300/mm³. Die Temperatur erreichte nur am 2. Tage 37,7° C. Komplikationen von seiten der Nieren wurden nicht beobachtet.

Fall 5: (Anna R., Krankenbl.-Nr. 4045/60) war eine 44jähr. Frau mit einer endogenen Depression, die nur wenige Schluck Essigessenz getrunken hatte. Sie hatte unmittelbar darauf reichlich Milch erhalten und war ½ Tag später auf Umwegen zu uns gekommen. Kollapserscheinungen ließen sich auch anamnestisch nicht objektivieren, die Verätzungsfolgen am Rachen waren sehr diskret. Die Leukozyten erreichten maximal 13 250/mm³, die Temperaturen in den ersten Tagen 37,9° C. Rest-N und Serum-Bilirubin blieben normal. Komplikationen traten nicht auf.

Im letzten Fall handelte es sich wiederum um eine schwere Essigsäure-Intoxikation, die nach den bisher gewonnenen Erfahrungen bereits im Frühstadium besonders intensiv behandelt wurde. Eine akute Niereninsuffizienz blieb trotz schwerster Hämolyse aus, jedoch verstarb der Patient mehrere Tage später im Herz- und Kreis-

* Herrn Prof. Dr. K. Lennert danken wir für die freundliche Überlassung der Obduktionsberichte über die Fälle 2, 3 und 6 auch an dieser Stelle herzlich.

Dieser 6. Fall (Adalbert E., Krankenbl.-Nr. 1349/62) war ein 40jähr. Mann mit einer vor längerer Zeit erworbenen Blindheit, der in suizidaler Absicht 2 Tassen Essigessenz getrunken hatte. Ein unbekannter Teil davon wurde erbrochen und möglicherweise auch aspiriert. Der Patient wurde knapp 2 Stunden später in die hiesige HNO-Klinik eingeliefert, wo eine Tracheotomie durchgeführt und zunächst 500 ml 5%ige Na HCO_3-Lösung i.v. infundiert wurden. Nach einer weiteren Stunde kam der Pat. zu uns, wo die Erkrankung anfangs den in Abb. 1 dargestellten Verlauf nahm. Aus dieser Abbildung ergeben sich gleichzeitig die wichtigsten, während des 1. Stadiums des Prozesses erhobenen Befunde. Das Serum-Hämoglobin betrug zu Beginn 1900 mg%, das pH im arteriellen Blut 7,30, das Urin-pH 6,45. Der anfänglich erheblich vertieften Atmung folgte nach Applikation von insgesamt 85 g Na HCO_3 i.v. eine apnoische Phase, die eine vorübergehende Poliomat-Beatmung erforderte. Unter reichlicher i.v. Infusion von Lävulose anstelle von Na HCO_3 schied der Patient laufend Urin aus, der von vornherein mit einem Dauer-Katheter aufgefangen wurde. Einer erneuten vertieften Atmung schloß sich nach i.v. Infusion von 7,5 g Na HCO_3 eine passagere Cheyne-Stokes'sche Atmung an, worauf das Natriumbicarbonat wieder durch Lävulose i.v. ersetzt wurde. Eine 13 Stunden post intoxicationem erstmals aufgetretene schwere Hämoptoe führte zu einem bedrohlichen Blutdruckabfall, der jedoch durch Volumenauffüllung und sonstige therapeutische Maßnahmen (vgl. Abb. 1) rasch aufgefangen werden konnte. Das pH im arteriellen Blut hatte sich zu dieser Zeit normalisiert (7,40).

Das Verhalten von Prothrombin-Komplex (Prothrombin, Faktor V/VI und VII) und Thrombelastogramm (TEG) in dieser initialen Phase der Erkrankung ergibt sich aus Abb. 2. Sie läßt erkennen, daß die Faktoren des Prothrombin-Komplexes in den ersten Stunden mehr oder weniger erheblich abfielen, wobei das Accelerator-Globulin ca. 5 Stunden post intoxicationem mit 5% einen exzessiv niedrigen Wert erreichte, um sich im weiteren Verlauf zunächst rasch zu normalisieren. Besonders bemerkenswert war das Verhalten des TEG, das während der 1. Phase des Prozesses bis zu seiner Normalisierung laufend registriert wurde (Abb. 3). Ein wesentliches Absinken der Blutplättchen konnte nicht beobachtet werden.

Abb. 4 zeigt, daß der Urin 4 Stunden nach der Vergiftung ausschließlich Oxy-Hämoglobin, dagegen kein Met-Hämoglobin enthielt. Von da ab findet sich ein praktisch linearer Anstieg der Met-Hämoglobin-Konzentration bei gegensätzlichem Verlauf des Uringehaltes an Oxy-Hämoglobin*.

Nach Überwindung des akuten Stadiums blieb der Kreislauf in den folgenden Tagen unter einer der Ausscheidung angepaßten Flüssigkeitseinfuhr ausgeglichen. Das vorübergehend abgefallene Serum-Kalium (vgl. Abb. 1) konnte durch entsprechende Substitution bald wieder normalisiert werden. Die Leukozyten erreichten in den beiden ersten Tagen maximal 33 000 mm³, die Temperatur schwankte in den ersten 4 Tagen um 38° C rektal. Das Serum-Bilirubin stieg am 2. Tage auf 2,9 mg%. Der Fermentgehalt des Serums ergab zur gleichen Zeit folgende Werte: LDH 1095 I. U., GOT = 300 I. U., GPT = 219 I. U., alkalische Phosphatase = 8,5 I. U. Die Laktatdehydrogenase (LDH) fiel nach weiteren 2 Tagen auf 758 I. U. ab. Der Rest-N blieb während der ganzen Zeit im Normbereich. Der Urin enthielt, nach einer anfänglichen leichten Proteinurie, nach 3 Tagen kein Eiweiß mehr, jedoch gelegentlich granulierte Zylinder sowie einzelne Erythrozyten.

* Herrn Doz. Dr. B. Hess sei für die betreffenden Bestimmungen (Cyan-Methode) auch an dieser Stelle herzlich gedankt.

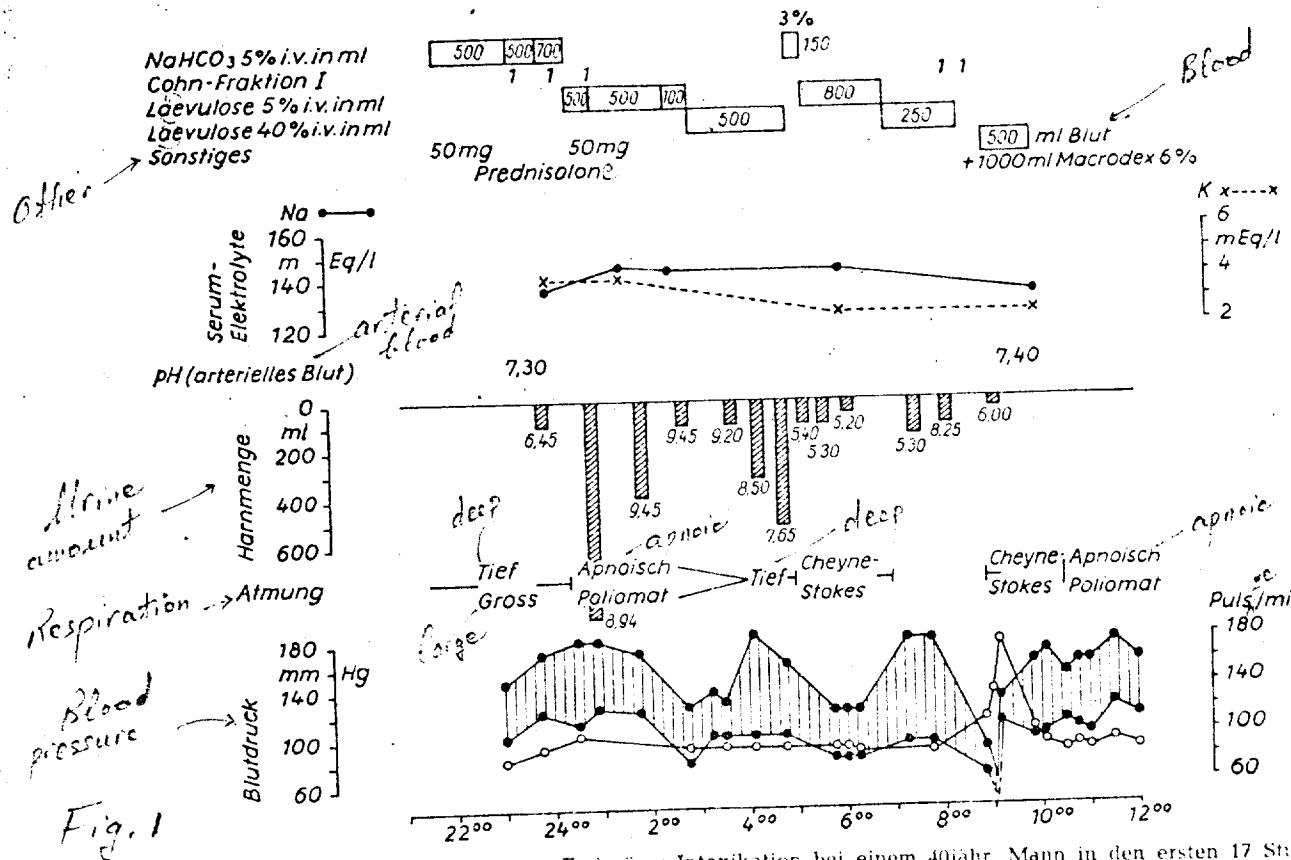

Abb. 1: Verlauf eines Falles von schwerer Essigsäure-Intoxikation bei einem 40jähr. Mann in den ersten 17 Stunden. Die Zahlen unter den Säulen, die die Harnmengen angeben, bedeuten das pH des Urins. Weitere Erläuterung siehe Text

Eine röntgenologische Untersuchung des Thorax 12 Stunden ante exitum ergab teilweise konfluierte bronchopneumonische Herde in beiden Unterfeldern sowie im linken Mittelfeld. Trotz einer von vornherein durchgeführten intensiven antibiotischen Behandlung (Chloramphenicol) entwickelten sich zu dieser Zeit plötzlich hyperpyretische Temperaturen, die sich therapeutisch nicht mehr beeinflussen ließen, so daß der Patient am 5. Tag nach der Intoxikation im Herz- und Kreislaufversagen ad exitum kam.

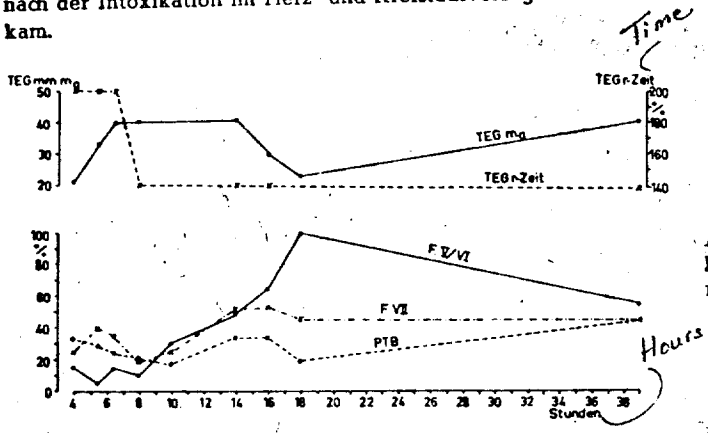

Abb. 2: Verhalten von Thrombelastogramm (TEG) und Prothrombin-Komplex (PTB = Prothrombin, F. VII = Faktor VII, F. V/VI = Faktor V/VI) bei einem Fall von schwerer Essigsäure-Intoxikation in der initialen Phase der Erkrankung. Weitere Erklärung siehe Text

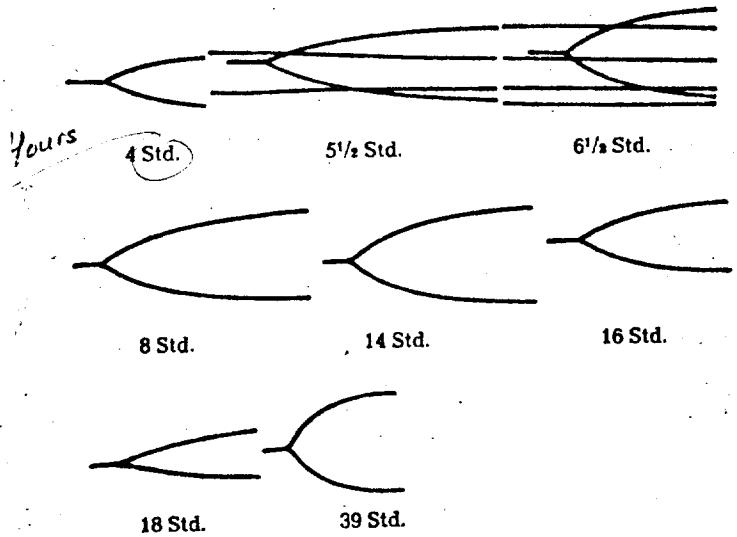

Abb. 3: Verhalten des Thrombelastogramms 4—39 Stunden nach peroraler Einnahme von 2 Tassen Essigessenz bei einem 40jähr. Mann

Autoptisch fanden sich hochgradige, bis zum Antrum ventriculi reichende Schleimhautverätzungen sowie eine schwere pseudomembranöse nekrotisierende Entzündung des Larynx, der Trachea und der Bronchien beiderseits, ferner ausgedehnte Pankreasfettgewebsnekrosen und eine starke, vorwiegend azinozentrale, stellenweise aber auch diffuse, mittel- bis großtropfige Leberzellverfettung mit kleinherdigen Leberzellnekrosen. Außerdem bestanden multiple, teils fleck-, teils punktförmige Blutungen in der Pleura visceralis beiderseits, ein rechtsseitiger hämorrhagischer Pleuraerguß (300 ml) und fleckförmige subendokardiale Blutungen in der Ausflußbahn der rechten Herzkammer. Die histologische Untersuchung der Nieren zeigte eine geringgradige hämoglobinurische Nephrose sowie eine diffuse feintropfige Verfettung der Harnkanälchenepithelien.

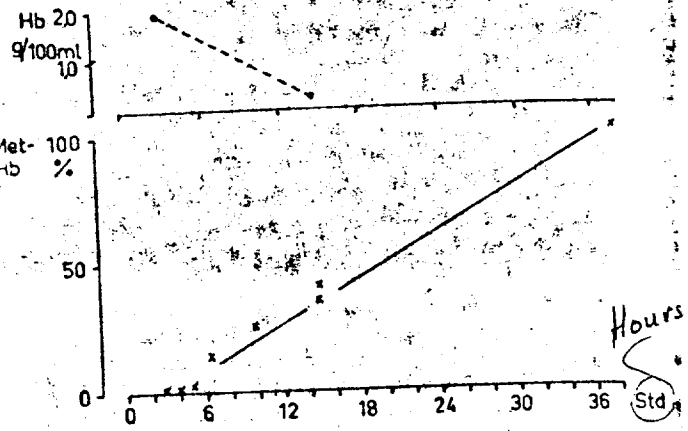

Abb. 4: Gehalt des Urins an Oxy-Hämoglobin (= Hb) und Met-Hämoglobin (= Met-Hb) in den ersten anderthalb Tagen nach peroraler Einnahme von 2 Tassen Essigessenz bei einem 40jähr. Mann. Weitere Erklärung siehe Text

Besprechung

Aus der Schilderung dieser 6 Patienten mit Essigessenz-Intoxikation, die in den letzten 3 Jahren an unserer Klinik beobachtet wurden, ergibt sich die ganze symptomatologische Skala dieses Krankheitsbildes. Die leichten Fälle (= Fall 4 und 5) werden beherrscht von den lokalen Verätzungsfolgen, ohne daß es dabei, wenn überhaupt, zu einer wesentlichen intravasalen Hämolyse kommt. Die betreffenden Schleimhautläsionen heilen relativ schnell ab und hinterlassen, soweit sich nach den vorliegenden Beobachtungen beurteilen läßt, keine bleibenden Residuen. Bei den schweren Vergiftungen findet sich darüber hinaus einerseits eine mehr oder weniger ausgeprägte Azidose (Fall 3 und 6), andererseits eine unter Umständen hochgradige Hämolyse (Fall 2, 3 u. 6). Auf letztere wurde bereits von verschiedenen Seiten (18, 22, 25, 26, 33/34, 40, 42, 50 53, 55, 58, 59, 61, 62) hingewiesen, während die Azidose von klinischer Seite nur vereinzelt (19, 58) erwähnt wird. Im Initialstadium des Prozesses besteht außerdem, in Übereinstimmung mit verschiedenen Autoren (6, 22, 34, 40, 48, 50, 64), ein mehr oder weniger hochgradiger Schock (Fall 1—3). Das weitere klinische Bild wird, sofern es nicht gelingt, die azidotische Stoffwechsellage und/oder den Schock therapeutisch rechtzeitig zu beeinflussen, bestimmt von dem typischen Bilde eines akuten Nierenversagens (Fall 1 und 2), dessen Problematik bereits andernorts besprochen wurde (26).

Die Pathogenese von Schock, Azidose und Hämolyse nach peroraler Einnahme von Essigessenz ist zweifellos ein recht komplexes Problem, auf das hier nur in großen Zügen eingegangen wird, da die weitere Klärung durch tierexperimentelle Untersuchungen (8) erfolgen soll. Es ist, in Anlehnung an Untersuchungen von Schibkow (52), anzunehmen, daß bereits die unmittelbare Ätzwirkung der Essigessenz auf die Schleimhäute der Mundhöhle sowie des oberen Intestinaltraktes zu einem Blutdruckabfall führen kann, der durch das gelegentlich sehr frühzeitig einsetzende Bluterbrechen, auf das schon Gerhartz (16), Hitzig (32), Kärber (24) Romeik (48) und Schäffer (50) aufmerksam machten noch akzentuiert wird. Zumindest in einem Teil der Fälle dürften diese Faktoren jedoch nicht ausreichen, um d

weitere Entwicklung nachhaltig zu bestimmen. Das wesentlichste pathogenetische Prinzip ist dann die mehr oder weniger ausgeprägte Azidose, von der man sich vorstellen kann, daß sie auf verschiedene Weise zu einem akuten Nierenversagen führt (Abb. 5): Die eine

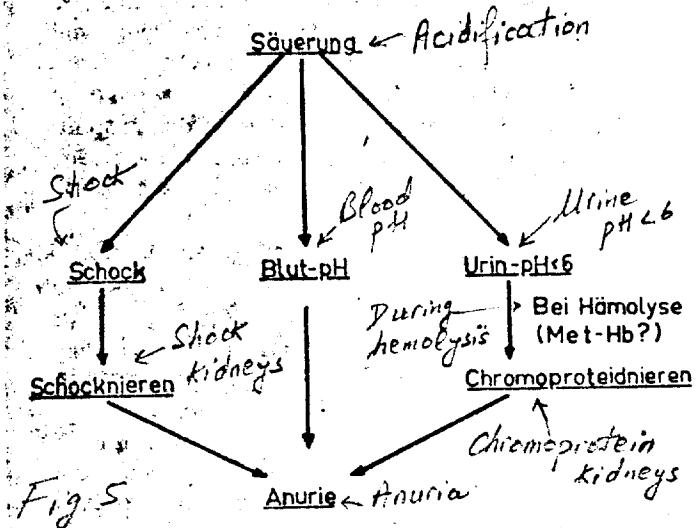

Abb. 5: Schematische Darstellung der möglichen pathogenetischen Wege, auf denen die Azidose bei der Essigsäure-Intoxikation zu einem akuten Nierenversagen führen kann. Weitere Erläuterung siehe Text

Möglichkeit ist die direkte Auslösung oder auch Aggravation eines Schocks mit konsekutiver Entwicklung von Schocknieren. Da jedoch nach tierexperimentellen Untersuchungen von Meesmann et al. (37) eine Säuerung allein, d. h. ohne wesentliche gleichzeitige hämodynamische Rückwirkungen, ebenfalls ein Versagen der Nierenfunktion zur Folge haben kann, ist auch ein solcher Mechanismus beim Zustandekommen der Anurie bzw. Oligurie zu diskutieren. Schließlich bliebe noch die Möglichkeit, daß eine schwere Azidose in Verbindung mit einer intravasalen Hämolyse zum Auftreten von Chromoproteidnieren führt. Voraussetzung für das Wirksamwerden einer derartigen pathogenetischen Kette scheint nach den experimentellen Arbeiten von Bing (2) und anderen (weitere Literatur: 47) allerdings die gleichzeitige Entstehung von Met-Hämoglobin zu sein, es sei denn, daß sich die intravasale Hämolyse mit einem Schock kombiniert und auf diese Weise zur Ausbildung einer Hämolyseniere Anlaß gibt. Dieser Mechanismus, auf den wir kürzlich besonders hingewiesen hatten (28), zeigt, daß das Zusammentreffen der verschiedenen, hier erörterten pathogenetischen Faktoren die Regel sein dürfte, wobei die Abgrenzung der einzelnen Teilkomponenten im Einzelfall wahrscheinlich kaum möglich ist.

Die im **Initialstadium** der schweren Essigsäure-Intoxikation auftretende Azidose spielt vermutlich auch beim Zustandekommen der Hämolyse eine wesentliche Rolle. Als ursächlicher Faktor ist in diesem Zusammenhang jedoch auch die Lipophilie der Essigsäure in Betracht zu ziehen, ohne daß sich ihr Anteil an der Pathogenese des hämolytischen Syndroms auf Grund der hier mitgeteilten Erfahrungen am Menschen genauer präzisieren ließe. Das Gleiche gilt letzten Endes auch für die Ursache der Azidose, worauf erst im Rahmen experimenteller Untersuchungen näher eingegangen werden soll.

Unsere kürzliche Feststellung, daß es im Rahmen des hämolytischen Prozesses bei der Essigsäure-Intoxikation zum Auftreten von Oxy- und nicht von Met-Hämoglobin komme (28), bedarf nach den jetzt vorliegenden Befunden einer Korrektur bzw. besser gesagt einer Ergänzung. Diese Behauptung, die im Gegensatz zu den Angaben von Kärber (24), Kogler (26) sowie Strzyzowski und Nicod (59) zu stehen schien, beruhte auf den spektrophotometrischen Untersuchungen an unserem 3. Fall und resultierte, wie die Ergebnisse des 6. Falles (Abb. 4) zeigen, seinerzeit offenbar daraus, daß im 3. Fall nur das Anfangsstadium des Geschehens erfaßt wurde. Abb. 4 läßt nämlich erkennen, daß sich das in der initialen Phase der Intoxikation tatsächlich ausschließlich vorhandene Oxy-Hämoglobin im weiteren Verlauf mehr und mehr in Met-Hämoglobin umwandelt, so daß der Urin etwa 14 Stunden nach Beginn der Erkrankung praktisch nur noch Met-Hämoglobin enthält. Auf diese Weise erklären sich wahrscheinlich die in der Literatur enthaltenen diesbezüglichen Widersprüche: In den Fällen, in denen man nur Oxy-Hämoglobin fand (20, 50, 55), wurden offenbar lediglich die frühen Stadien der Intoxikation, in jenen Fällen, bei denen Met-Hämoglobin nachweisbar war (24, 26, 59), anscheinend vorwiegend die späteren Phasen erfaßt. Es erscheint damit jedenfalls sicher, daß es sich bei der Essigessenz nicht um einen primären Met-Hämoglobin-Bildner handelt, sondern daß dieses Oxydationsprodukt des Hämoglobins bei der Essigsäure-Intoxikation erst allmählich auf Grund biologischer Reaktionsabläufe in Erscheinung tritt.

Von besonderem Interesse ist ferner die bei der schweren Essigsäure-Vergiftung zu beobachtende **allgemeine hämorrhagische Diathese**, auf die wir an Hand des 3. Falles bereits andernorts (28) kurz hingewiesen hatten.

Dieser Fall zeigte unmittelbar vor Einsetzen des Blutdruckabfalles außer einer Abnahme des Prothrombin-Komplexes (Prothrombin, Faktor V/VI und VII) eine erhebliche Hypofibrinogenämie mit Thrombozytopenie. Das Verhalten von Blutplättchen, Thrombelastogramm (Abb. 2 und 3) und Prothrombin-Komplex (Abb. 2) in den ersten anderthalb Tagen der Vergiftung konnte nunmehr in einem weiteren Fall (= Fall 6) kontinuierlich verfolgt werden. Hierbei waren in den ersten 10 Stunden post intoxicationem die Faktoren des Prothrombin-Komplexes, insbesondere das Accelerator-Globulin (Faktor V/VI), stark vermindert, während die Thrombozyten annähernd im Normbereich blieben. Der Faktor V hatte sich nach 18 Stunden zunächst völlig normalisiert, sank aber nach weiteren 21 Stunden erneut auf 55% ab. Eine Normalisierung von Prothrombin und Faktor VII konnte während dieses Zeitraumes nicht beobachtet werden. Die TEG-Kurven waren zu Beginn der Intoxikation stark verschmälert. Wenige Stunden später hatte die maximale Amplitude des TEG normale Werte erreicht, um nach 16 Stunden allerdings nochmals abzunehmen. Die anfänglich verlängerte Gerinnungszeit war schon 4 Stunden nach Therapiebeginn endgültig normalisiert.

Eine Deutung dieser Befunde ist noch nicht möglich. Durch entsprechende Pufferung unserer Gerinnungsansätze ist es ausgeschlossen, daß methodische Fehler auf Grund der Säuerung diese Werte entstehen ließen. Eine Klärung wird zur Zeit von Lasch und Mitarb. herbeigeführt. Nach unseren Erfahrungen kann schon jetzt gesagt werden, daß diesen Gerinnungsveränderungen bei der Beurteilung der Schwere der Intoxikation eine wesentliche Bedeutung zukommt.

Die hier diskutierten Änderungen des Gerinnungspotentials betreffen allerdings nur die initiale Phase des Prozesses. In späteren Stadien auftretende

Verminderungen des Prothrombin-Komplexes, wie sie in Fall 1 sowie andeutungsweise auch in Fall 6 beobachtet wurden, dürften auf der im Laufe der Intoxikation bei genügend langer Überlebensdauer sich entwickelnden mehr oder weniger schweren Leberschädigung beruhen. Eine solche zusätzliche Organläsion, auf die bereits mehrere Autoren hingewiesen haben (17—19, 35, 39—41), ließ sich — bei Berücksichtigung der Ergebnisse von Gerlach et al. (18) — im 6. Fall durch den auffallend kleinen LDH GOT-Quotienten bereits intra vitam wahrscheinlich machen und pathologisch-anatomisch bestätigen. Im gleichen Sinne sprechen die klinischen Daten des 1. Falles, wenngleich hier wegen des chronischen Potatoriums ein präexistenter Leberparenchymschaden angenommen werden muß. Immerhin fällt auf, daß bei diesem Patienten während der mehrmonatigen Beobachtung die Lebergröße zurückging und die Leberfunktionsproben, vor allem der Bromthalein-Test, teilweise eine deutliche Tendenz zur Besserung zeigten. Die Ursache des Zustandekommens eines Leberzellschadens bei Fällen von Essigsäure-Intoxikation, die mindestens mehrere Tage am Leben bleiben, wird allerdings weiteren Untersuchungen vorbehalten bleiben müssen.

Die Frage nach einer **adäquaten Behandlung der Essigsäure-Vergiftung** war von uns (28) bislang im wesentlichen nur im Hinblick auf das stets drohende akute Nierenversagen erörtert worden. An Hand des 3. Falles war jedoch bereits darauf hingewiesen worden, daß die bei schweren Intoxikationen unter Umständen rasch einsetzende, hochgradige Azidose mit allen ihren Konsequenzen besondere therapeutische Maßnahmen erfordert. Ihr oberstes Ziel wird sein, den durch Schock, Azidose und Hämolyse ausgelösten Circulus vitiosus so früh wie möglich zu durchbrechen, um damit nicht zuletzt die Entwicklung einer Niereninsuffizienz zu vermeiden. Auf Grund der inzwischen gewonnenen Erfahrungen konnte dies in dem letzten von uns beobachteten Fall erreicht werden (Abb. 1). Ausgehend von den in den vorausgegangenen Betrachtungen angestellten Überlegungen (vgl. Abb. 5) scheint vor allem eine maximal dosierte intravenöse Applikation von Natriumbicarbonat von entscheidender Bedeutung zu sein. Der genannte Fall lehrt im übrigen, daß die Therapie eine laufende Kontrolle der Atmung erfordert. Eine sofort vorgenommene Tracheotomie mit der Möglichkeit einer künstlichen Beatmung stellt deshalb eine Conditio sine qua non dar. Bei Atemstörungen (vgl. Abb. 1) empfiehlt sich, die erwähnte Alkalisierung vorübergehend abzubrechen. In diesem Fall ist die Fortsetzung der Infusionsbehandlung mit mehr oder weniger hochprozentiger Lävulose solange dringend angezeigt, als die sorgfältig überwachte Diurese eine solche Flüssigkeitszufuhr erlaubt. Auf diese Weise konnten im hier diskutierten 6. Fall trotz erheblicher Hämoglobinurie die kritischen ersten 24 Stunden überwunden werden. Andernfalls gelten selbstverständlich die Regeln für die Therapie des akuten Nierenversagens. Läßt sich trotz allem ein Schock nicht vermeiden, so wird man u. a. daran denken müssen, daß bei azidotischer Stoffwechsellage in einem gewissen Bereich eine verminderte Ansprechbarkeit der Arteriolen auf Angiotensin besteht, so daß man gegebenenfalls auf einen Arterenol-Dauertropf zurückgreifen muß (36).

Insgesamt empfiehlt sich also nach den hier vorliegenden Erfahrungen für die schwere Essigsäure-Intoxikation das **folgende therapeutische Vorgehen:**

1. Innerhalb der ersten 10—15 Minuten: Magenspülung und anschließend Mg $(OH)_2$ (38); sonst bzw. danach:
2. Tracheotomie; gleichzeitig bzw. möglichst umgehend:
3. 5%iges Natriumbicarbonat i.v. (unter laufender Kontrolle der Atemtiefe); gegebenenfalls (vgl. oben):
4. 5%ige Lävulose i.v. (je nach Diurese, die sorgfältig überwacht werden muß). Bei Absinken der Harnmenge entweder erneut Na HCO_3 i.v. oder 40%ige Lävulose i.v. (osmotische Diurese);
5. Bei schon bestehendem Schock zusätzlich die übliche Schocktherapie (Hypertensin- oder Arterenol-Dauertropf, vgl. oben);
6. Bei Abnahme der Harnausscheidung die allgemein gültigen Regeln der Behandlung des akuten Nierenversagens.

Das weitere Schicksal des Patienten wird, wie der 6. Fall zeigt, natürlich nicht zuletzt von dem Ausmaß der Verätzungen bestimmt. Es wird außerdem weiterer Erfahrungen bedürfen, um zu entscheiden, ob das bei der Essigsäure-Vergiftung stets drohende akute Nierenversagen durch das hier skizzierte Vorgehen wenigstens in der Mehrzahl der Fälle verhindert werden kann.

Alles in allem scheint die Kenntnis dieser Intoxikation und ihrer Behandlungsmöglichkeiten zunehmendes Interesse zu beanspruchen, da sie neuerdings offenbar häufiger vorkommt, als man auf Grund der relativ spärlichen Literaturangaben annehmen sollte.

Zusammenfassung

Es wird über insgesamt 6 Fälle von Intoxikation nach peroraler Einnahme von Essigessenz berichtet, von denen 2 leicht und komplikationslos, 4 dagegen ausgesprochen schwer verliefen. Von letzteren zeigten 2 das typische Bild eines akuten Nierenversagens, wobei 1 mit Hilfe der extrakorporalen Hämodialyse bzw. einer Peritonealdialyse gerettet werden konnte. Ein weiterer Patient verstarb innerhalb von 10 Stunden unter dem Bild einer schwersten Azidose und Hämolyse im Schock, während im letzten Fall unter adäquater Therapie eine Niereninsuffizienz trotz ausgeprägter Azidose und Hämolyse verhindert werden konnte.

Das klinische Bild der Erkrankung wird unter besonderer Berücksichtigung der Pathogenese von Azidose, Hämolyse und gegebenenfalls Schock sowie deren möglichen Konsequenzen (akutes Nierenversagen) eingehend besprochen. Hieraus resultieren die für die Behandlung der initialen Phase der Essigsäure-Intoxikation sinnvollen therapeutischen Maßnahmen, durch die sich, wie die Erfahrung lehrt, die Entwicklung eines akuten Nierenversagens unter Umständen verhindern läßt.

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ACTION OF ACETIC ACID ON FOOD SPOILAGE MICROORGANISMS[1]

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This investigation was initiated with the purpose of studying the effect of acetic acid on certain microorganisms related to food spoilage with the hope of securing results of practical value. The main reasons for the use of acetic acid as a food preservative are its toxicity value, commercial availability, and low cost.

The toxic effect on microorganisms of an increased hydrogen-ion concentration in the substrate is well established. Acetic acid and certain other organic acids appear to have a toxicity in excess of that which could possibly be due to the pH alone. The consensus of authorities seems to favor the theory that it is the undissociated molecule which is toxic. Kahlenberg and True (1896) found that the undissociated acetic acid molecule was toxic. Winslow and Lockridge (1906) found that acetic and benzoic acids were fatal to typhoid and colon bacilli at a strength at which these acids were but slightly dissociated. Wolf and Shunk (1921) concluded that the hydrogen-ions alone were not responsible for the toxicity of acetic acid. Bach (1932) states that at an interval where the pH has no importance, the undissociated part of the lactic acid is the active factor, although generally the hydrogen-ions control the antiseptic effect. In another paper (1932a) the same investigator suggested that the antiseptic action of formic, acetic, propionic, and butyric acids was connected with their influence on surface tension. Foster (1920) also contributed to the idea that the toxic effect was due to the

[1] Contribution No. 352, Massachusetts Agricultural Experiment Station. Presented at the Third International Congress for Microbiology, New York City, September 5, 1939.

whole molecule rather than to hydrogen-ions alone. Kirby, Frey, and Atkin (1935, 1936) found that acetic acid, either chemically pure or as vinegar, had a marked influence on the growth of bread molds, particularly *Aspergillus niger*. These investigators observed that at the same pH, greater inhibition was obtained with an increasing concentration of acetic acid. Collett (1919, 1921) stated that, although the hydrogen-ions are important in solutions of equal pH, butyric and acetic acids were more toxic than hydrochloric and other strong acids. Tekelenberg (1927) demonstrated that the alkali salts alone of several organic acids had no marked bactericidal effect. Cohen and Clark (1919) concluded that the limiting pH is a zone rather than a value and the chief effect of reaction in growth rate studies seems to be confined to the extremes. They suggest that the toxic action of acetic acid may be due to the free acetate radical exerting a synergic effect on the disinfecting power of the hydrogen ion.

Kolthoff (1925) stated that the growth of bacteria in cultures is halted not by the hydrogen-ions, but by the formation of undissociated acids. Bach (1932) has stated that generally the hydrogen-ions control the antiseptic effect but that the undissociated part of lactic acid is the active factor when the pH value is such as to be unimportant.

EXPERIMENTAL

The studies in this paper include: (1) A determination of the concentrations of acetic acid in broth media which will destroy and inhibit the growth of the test organisms during the incubation period; (2) An investigation of the effect of acetic acid on the thermal death points of the test organisms; and (3) A comparison of acetic, lactic, and hydrochloric acids as to their inhibitory and toxic properties on a typical yeast, mold, and bacterium. Hydrogen-ion activities as well as total titratable acidity are considered in these experiments.

INDEX ORGANISMS

The test microorganisms used in this investigation were: *Salmonella aertrycke, Staphylococcus aureus, Phytomonas phaseoli,*

Bacillus cereus, Bacillus mesentericus; Saccharomyces cerevisiae Lister; and *Aspergillus niger.*

METHODS

Inhibiting and lethal concentrations of acetic acid were determined by a somewhat modified phenol-coefficient technic whereby known quantities of acid and medium were mixed in a series of test tubes, inoculated and incubated at suitable temperatures. The comparative effects of acetic, lactic, and hydrochloric acids on the total destruction and reduction of numbers of bacteria and yeast were determined by a 15-minute contact of the test organism in an acid solution followed by a standard quantitative plating on nutrient agar for the bacteria and on glucose agar for the yeast. The mold was cultivated in flasks of glucose broth and after the incubation period the mat was carefully washed, dried and weighed. Thermal death points were studied in test tubes in media whose hydrogen-ion concentrations were adjusted by acetic acid. Suspensions of spore-forming bacteria contained spores as well as vegetative cells. Inoculations of all suspensions were made by means of a standard four-millimeter loop.

RESULTS

Table 1 summarizes the data obtained in determining concentrations of acetic acid which inhibited growth and those that were lethal to the organisms in tubes of broth. The growth of *Salmonella aertrycke* in nutrient broth, containing acetic acid, was inhibited at pH 4.9 but the organism was still viable. At pH 4.5 this organism was destroyed. *Aspergillus niger* was slightly less resistant to the action of acetic acid than was the yeast. Both yeast and mold were markedly more resistant to the acid than were any of the bacteria studied. Where the inhibiting and lethal acidities were the same, slight differences might have been shown had the pH increments been smaller.

Thermal death point studies

Thermal death point studies on microorganisms are of considerable value, especially to the canner in determining processing times and temperatures for the preservation of various food

products. The addition of a small amount of acetic acid may aid in reducing the time or temperature required to sterilize the product commercially. Table 2 is a summary of results obtained when the pH of the broth was adjusted with acetic acid. The effect of an acid reaction in reducing the thermal death temperature was most marked in the case of the aerobic spore-formers, *Bacillus cereus* and *B. mesentericus*. The addition of small amounts of acetic acid to the medium did not affect the thermal death points of the yeast or mold.

TABLE 1

Inhibiting and lethal acetic acid concentrations for microorganisms

ORGANISM	INHIBITING pH*	INHIBITING ACIDITY	LETHAL pH†	LETHAL ACIDITY
		per cent		per cent
Salmonella aertrycke	4.9	0.04	4.5	0.09
Staphylococcus aureus	5.0	0.03	4.9	0.04
Phytomonas phascoli	5.2	0.02	5.2	0.02
Bacillus cereus	4.9	0.04	4.9	0.04
Bacillus mesentericus	4.9	0.04	4.9	0.04
Saccharomyces cerevisiae	3.9	0.59	3.9	0.59
Aspergillus niger	4.1	0.27	3.9	0.59

* The pH at which no visible growth occurred yet the microorganism remained viable.

† The pH at which total destruction took place.

Comparison of acetic, lactic, and hydrochloric acids

To determine whether the degree of toxicity of added acid was due to the hydrogen-ion activity, to the organic or inorganic nature of the acid, or to some factor peculiar to acetic acid, experiments were conducted to compare the effects of acetic, lactic, and hydrochloric acids. *Salmonella aertrycke*, *Saccharomyces cerevisiae* Lister, and *Aspergillus niger* were used. The results with *Salmonella aertrycke* are shown in table 3. This organism was inhibited in broth at pH 4.9 and killed in broth at pH 4.5 containing 0.04 and 0.08 per cent acetic acid, respectively. *Salmonella aertrycke* grew in broth with a pH 4.5, having a lactic acid content of 0.06 per cent and was inhibited and killed when the lactic acid was increased to 0.12 per cent with a pH

of 4.0. An acidity of 0.03 per cent of hydrochloric acid with a pH value of 4.0 inhibited growth. Toxicity and inhibition in the case of hydrochloric acid seem to be due to the hydrogen-ion concentration and not to the whole molecule as in the case of acetic acid. Lactic acid is the least effective in inhibiting growth of *S. aertrycke* under the conditions of this experiment.

TABLE 2

Effect of pH of the medium on the thermal death point of microorganisms, exposure period, 10 minutes

Reaction adjusted by acetic acid

ORGANISM	pH VALUE	LETHAL TEMPERATURE	
		°C.	°F.
Salmonella aertrycke	6.6	55	131
	5.0	50	122
Staphylococcus aureus	6.6	65	149
	5.5	60	140
Phytomonas phaseoli	6.6	55	131
	5.7	50	122
Bacillus cereus	6.6	100	212
	5.5	60	140
Bacillus mesentericus	6.6	100	212
	5.5	60	140
Saccharomyces cerevisiae	6.8	60	140
	4.5	60	140
Aspergillus niger	6.8	60	140
	5.0	60	140
	4.5	60	140

Table 4 shows the results obtained with yeast. Cells of *Saccharomyces cerevisiae* were destroyed in glucose broth tubes of pH 3.9 or lower with an acetic acid content of 0.59 per cent or more. The toxic limit of lactic acid for this yeast was considerably higher, being between 1.54 and 3.08 per cent. Again, less hydrochloric acid destroyed the test organism but the hydro-

gen-ion activity in the limiting tube with hydrochloric acid was 2.2 as compared with the limiting pH of 3.9 when acetic acid was used.

TABLE 3

Comparative inhibitory effect of acetic, lactic, and hydrochloric acids in nutrient broth on the growth of Salmonella aertrycke

MEDIUM	ACIDITY OF BROTH		COMPLETE INHIBITION OF GROWTH, 48 HOURS	TOTAL DESTRUCTION OF BACTERIA, 48 HOURS
	pH	Calculated total acidity		
		per cent		
Broth + acetic acid	4.0	0.33	−	−
	4.3	0.17	−	−
	4.5	0.08	−	−
	4.9	0.04	−	+
	5.2	0.02	+	+
	5.6	0.01	+	+
	5.7	0.005	+	+
Broth + lactic acid	3.2	0.46	−	−
	3.6	0.23	−	−
	4.0	0.12	−	−
	4.5	0.06	+	+
	5.0	0.03	+	+
	5.5	0.01	+	+
	6.0	0.007	+	+
Broth + HCl acid	1.5	0.27	−	−
	2.1	0.13	−	−
	3.1	0.07	−	−
	4.0	0.03	−	+
	4.7	0.02	+	+
	5.2	0.008	+	+
	5.8	0.004	+	+

The inoculation consisted of one loopful of a 48-hour broth culture of *Salmonella aertrycke*.

Table 5 shows that in acidity ranges similar to those of the yeast experiment, the spores of *Aspergillus niger* were killed when the hydrogen-ion concentration of the medium adjusted with acetic acid was pH 3.9 and the total acidity was 0.59 per cent. At pH values of 4.0 and 4.1 corresponding to 0.37 and 0.27 per cent acidity, respectively, the mold spores did not vegetate but

did maintain their viability. When the medium was adjusted with 0.27 per cent hydrochloric acid resulting in a pH of 1.6, the spores not only survived but also developed into vegetative mycelia. With this acid a pH value of 1.2 and an acidity of 0.38 per cent was required to prevent growth. *Aspergillus niger* tolerated

TABLE 4

Comparative inhibitory effect of acetic, lactic, and hydrochloric acids in glucose broth on the growth of Saccharomyces cerevisiae Lister

MEDIUM	ACIDITY OF BROTH		COMPLETE INHIBITION OF GROWTH, 48 HOURS	TOTAL DESTRUCTION OF YEAST CELLS, 48 HOURS
	pH	Calculated total acidity		
		per cent		
Broth + acetic acid	3.6	1.18	−	−
	3.7	0.74	−	−
	3.9	0.57	−	−
	4.0	0.37	+	+
	4.4	0.18	+	+
	4.7	0.09	+	+
Broth + lactic acid	2.0	12.32	−	−
	2.2	6.16	−	−
	2.5	3.08	−	−
	2.8	1.54	+	+
	3.2	0.77	+	+
	3.4	0.39	+	+
Broth + HCl acid	1.6	0.27	−	−
	2.2	0.13	−	−
	3.3	0.07	+	+
	4.1	0.03	+	+
	4.8	0.02	+	+

The inoculation consisted of one loopful of a 48-hour broth culture of *Saccharomyces cerevisiae* Lister.

a relatively high lactic acid content in the medium, growing abundantly at 3.08 per cent. Here, the limiting pH of 2.2 was higher than that of the hydrochloric acid but lower than that of acetic acid. Because acetic acid was toxic at a lower hydrogen-ion activity and especially in the case of mold at a lower total acidity than either lactic or hydrochloric acid, the toxicity of

acetic acid is not entirely a function of pH but due more to the nature of the acid itself.

Comparative quantitative studies

The comparative effects of different concentrations of acetic, lactic, and hydrochloric acids on the extent of growth of *Sal-*

TABLE 5

Comparative inhibitory effect of acetic, lactic, and hydrochloric acids in glucose broth on the growth of Aspergillus niger

MEDIUM	ACIDITY OF BROTH		COMPLETE INHIBITION OF GROWTH, 48 HOURS	TOTAL DESTRUCTION OF MOLD, 48 HOURS
	pH	Calculated total acidity		
		per cent		
Broth + acetic acid	3.6	1.18	−	−
	3.7	0.74	−	−
	3.9	0.59	−	−
	4.0	0.37	−	+
	4.1	0.27	−	+
	4.4	0.18	+	+
	4.7	0.09	+	+
Broth + lactic acid	2.0	12.32	−	−
	2.2	6.16	−	−
	2.5	3.08	+	+
	2.8	1.54	+	+
	3.2	0.77	+	+
	3.4	0.39	+	+
Broth + HCl acid	1.2	0.38	−	−
	1.6	0.27	+	+
	2.2	0.13	+	+
	3.3	0.07	+	+
	4.1	0.03	+	+

The inoculation consisted of a loopful of a water suspension containing about 200 spores.

monella aertrycke, *Saccharomyces cereviseae*, and *Aspergillus niger* were studied. Table 6 shows that acetic acid was the most toxic acid to *S. aertrycke* on the basis of hydrogen-ion activity. It was slightly better than lactic acid at an equivalent pH value and was much more toxic than hydrochloric acid when in contact with the test organism for 15 minutes. It is true that less hydro-

chloric acid was required to increase the hydrogen-ion activity to its toxic limit than in the case of either lactic or acetic acids. However, both the latter acids were potent at a higher pH than the hydrochloric acid. The percentage reduction in numbers of

TABLE 6

Comparative effect of acetic, lactic, and hydrochloric acids on the survival of Salmonella aertrycke in water

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*bacteria per ml.*	*per cent*
Water + acetic acid	2.9	0.33	0	100.00
	3.1	0.17	420	98.83
	3.3	0.08	1,800	95.00
	3.5	0.04	3,600	90.00
	3.7	0.02	7,200	80.00
	3.9	0.01	13,900	61.39
Water + lactic acid	2.5	0.46	0	100.00
	2.7	0.23	0	100.00
	2.9	0.11	14	99.96
	3.0	0.06	200	99.44
	3.2	0.03	1,500	95.83
	3.4	0.01	6,000	83.33
	3.6	0.007	16,200	55.00
Water + HCl acid	1.3	0.27	0	100.00
	1.6	0.13	0	100.00
	1.9	0.07	0	100.00
	2.1	0.03	750	97.92
	2.4	0.02	12,000	66.67
	2.7	0.01	20,400	43.33
Water only	6.2	0.0	36,000	

The inoculation per 5 ml. of solution contained 120,000 bacteria.

surviving bacteria was calculated on the basis of the number of *S. aertrycke* present in the distilled water.

The effect of the three acids on the yeast, *S. cereviseae*, after a 15-minute contact period is shown in table 7. The difference in limiting acidities was much more marked with this organism

than with *S. aertrycke*. For complete destruction of the yeast cells 2.94 per cent acetic acid at a pH of 2.6 was required as compared with 5.25 to 10.49 per cent lactic acid at a pH value of about 1.7 and 0.92 per cent hydrochloric acid at a pH value of

TABLE 7

Comparative effect of acetic, lactic, and hydrochloric acids on the survival of Saccharomyces cerevisiae Lister *in water*

Contact period, 15 minutes

TYPE OF SOLUTION	ACIDITY OF SOLUTION		TOTAL COUNT	REDUCTION IN NUMBERS
	pH	Calculated total acidity		
		per cent	*cells per ml.*	*per cent*
Water + acetic acid	2.6	2.94	0	100.00
	2.8	1.47	90	91.00
	2.9	0.74	311	68.90
	3.1	0.37	450	55.00
	3.2	0.18	560	44.00
	3.4	0.09	690	31.00
Water + lactic acid	1.7	10.49	0	100.00
	1.8	5.25	9	99.10
	2.0	2.62	134	86.60
	2.1	1.31	340	66.00
	2.3	0.66	380	62.00
	2.6	0.33	370	63.00
Water + HCl acid	0.2	3.68	0	100.00
	0.6	1.84	0	100.00
	0.7	0.92	0	100.00
	1.1	0.46	146	85.40
	1.3	0.23	470	53.00
	1.6	0.12	470	53.00
Water only	6.2	0.0	1,000	

The inoculation per 5 ml. of solution contained 4500 cells.

0.7. There was some survival of yeast cells when the hydrogen-ion activity of the lactic acid solution was pH 1.8 and of the hydrochloric acid solution was 1.1. Here, although less lactic acid than acetic acid was required to give the same pH value, a smaller content of acetic than lactic acid was required for the

same degree of toxicity. A quantity of the strongly dissociated hydrochloric acid smaller than in the case of either of the other two weakly dissociated acids was required to reduce the number

TABLE 8

Comparative effect of acetic, lactic, and hydrochloric acids on the growth of Aspergillus niger of glucose broth

MEDIUM	INITIAL ACIDITY		FINAL ACIDITY		DRY WEIGHT
	pH	Titratable acidity	pH	Titratable acidity	
		per cent		*per cent*	*mgm.*
Broth + acetic acid	4.1	0.27	4.1	0.27	0.0
	4.3	0.21	4.3	0.21	17.3
	4.4	0.16	7.1	0.03*	162.0
	4.5	0.15	7.6	0.03	147.8
	4.7	0.10	7.6	0.03	133.3
	5.2	0.07	7.6	0.03	120.5
Broth + lactic acid	2.2	4.84	2.2	5.43	0.0
	2.3	4.16	2.3	4.56	16.7
	2.4	3.48	2.8	1.24	495.7
	2.5	2.46	3.2	0.24	486.3
	2.6	1.91	4.2	0.06	424.0
	2.7	1.80	7.1	0.04	375.4
	2.8	1.21	7.4	0.04	311.0
	3.1	0.72	7.6	0.04	206.0
Broth + HCl acid	1.3	0.35	1.3	0.36	0.0
	1.4	0.30	1.4	0.31	8.0
	1.6	0.25	1.6	0.26	17.5
	1.8	0.21	1.8	0.21	41.7
	2.3	0.12	7.5	0.04	163.8
	2.9	0.10	7.6	0.04	136.7
Broth control	6.8	0.03	7.6	0.04	128.1

The inoculation consisted of 0.1 ml. of a water suspension containing about 2000 spores.

* At final pH values of 7.0 or higher the small apparent acidity represents a blank which is not subtracted in this table.

of surviving cells but the toxic action of hydrochloric acid manifested itself at a relatively much lower pH value than that of the other two acids, especially acetic acid.

Plate 1 shows the comparative effects of acetic, lactic, and hydrochloric acids on the growth of *Aspergillus niger* in glucose broth. The data are summarized in table 8. Complete inhibition of this mold was caused by the addition of 0.27 per cent acetic acid, 4.84 per cent lactic acid, or 0.35 per cent hydrochloric acid to glucose broth. The limiting pH values were 4.1, 2.2, and 1.3 respectively. In other words, less acetic than either lactic or hydrochloric acids was required to prevent mold growth and the toxic limit when acetic acid was used was at a lower hydrogen-ion activity than that of the other two acids. There was some inhibition as indicated by only a slight growth when acetic or hydrochloric acids were present in a concentration of 0.21 per cent. At non-toxic concentrations, the mold readily utilizes lactic acid to aid in the development of a heavy, rubbery mat unlike those produced in the presence of non-toxic amounts of either acetic or hydrochloric acids. Within non-toxic limits those flasks which contained the more lactic acid had the heavier mold growth. In the lactic acid series it is also interesting to note that acidities of 3.48 and 2.46 per cent gave rise to mats of approximately the same weight. This is not surprising if the final acidities are deducted from the initial acidities to determine the amounts of acid utilized. Thus, with an initial acidity of 3.48 per cent and a final acidity of 1.24 per cent, 2.24 per cent lactic acid was utilized to yield a dry weight of 495.7 mgm. When the initial acidity was 2.46 per cent and the final acidity 0.24 per cent, 2.22 per cent lactic acid was utilized to yield a dry weight of 486.3 mgm. These figures are in accordance with other initial acidities and final yields of mold in this series.

Again it is evident that the toxicity of acetic acid to mold spores is a function of the nature of the acid, perhaps in addition to the hydrogen-ion activity. In the case of lactic acid, too, the toxicity seems to be due to more than the hydrogen-ion activity alone as the limiting pH value was higher in the lactic acid series than the limiting pH value of the hydrochloric acid series. However, the toxic principle of the lactic acid was far less potent toward spores of *A. niger* than the toxic factor of the acetic acid.

DISCUSSION AND CONCLUSIONS

The premise is fairly well established that an increase in acidity or in hydrogen-ion concentration inhibits the growth of, or even destroys, certain microorganisms and materially reduces their resistance to heat. The results of this investigation dealing mainly with the activity of acetic acid on certain microorganisms related to food spoilage confirm the findings of other investigators and cast some light on a somewhat obscure phenomenon: the peculiar toxicity of acetic acid.

It has been demonstrated that acetic acid in small amounts and at relatively high pH values proved toxic to representative aerobic bacteria and a yeast and mold. Lactic and hydrochloric acids were toxic to these same test organisms but at a higher total acid concentration or at a lower pH. The lethal activity at an unquestionably higher pH value than that obtained with hydrochloric acid is an indication that the effect of acetic acid is not due to the hydrogen-ion activity alone. The toxicity of acetic acid cannot be attributed to its general organic nature and slight ionization because lactic acid in larger quantities was required to effect the same toxicity despite the fact that a higher hydrogen-ion activity was observed with lactic acid than with an equivalent amount of acetic acid. This latter point is mentioned because it is true that the toxicity is increased at higher hydrogen-ion concentrations.

The results, in general, show that the hydrogen-ion activity cannot be entirely responsible for the toxicity of acetic acid. Many investigators are of the opinion that the lethal effect of the toxic organic acids is due, at least in part, to the undissociated molecule. However, the mechanism of inhibition is mainly a matter of conjecture. Wynne (1931) states that it is the accumulation of adsorbed substance which seems to exert some influence on the cell, but one can only speculate as to the essential physiological effect of this concentrated layer of adsorbed substance, resulting in the failure of the cell to function normally. That the influence of adsorbed acid is due to something more than the mere physical presence of a foreign substance is proved

by the fact that adsorption of the saturated paraffin series of fatty acids by bacteria is often possible without upsetting their metabolism.

Not only can acetic acid inhibit and destroy microorganisms when used in sufficiently high concentrations but it also aids materially in reducing the thermal death points of bacteria when present in sub-lethal concentrations. Many practical applications can be inferred from this phenomenon. The addition of a small amount of acid exerts more of a beneficial effect on the preservation of canned foods by lowering the thermal death point than by inhibiting the growth of the organism. Some of the major problems in the canning of foods are related to the high heat treatment necessary for the commercial sterilization of the so-called "non-acid" products. The addition of acetic acid is justified provided the appearance and flavor are not noticeably altered. The long accepted usage of vinegar as a common food component precludes any objection to this acid from a physiological standpoint.

SUMMARY

The toxic effect of vinegar upon certain microorganisms is usually attributed to its acetic acid content.

Acetic acid in nutrient broth inhibited the growth of various microorganisms related to food spoilage. The bacteria used did not grow in broth adjusted with acetic acid to pH 4.9. *Saccharomyces cerevisiae* did not grow at pH 3.9 and *Aspergillus niger* was inhibited at pH 4.1.

An increase in the hydrogen-ions resulted in a decrease of the thermal death points of the bacteria studied. The reduction in lethal temperature was more marked in the case of *Bacillus mesentericus* and *Bacillus cereus* than with the non-spore forming organisms. Thermal death points of the yeast and the mold were unaltered by the addition of small amounts of acetic acid.

Comparative studies showed acetic acid to be more toxic than either lactic or hydrochloric acid to *Salmonella aertrycke*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. These organisms were inhibited or destroyed at a higher pH value with acetic acid than with lactic or hydrochloric acids. The mold utilized relatively high amounts of lactic acid to develop a growth heavier than that obtained from the acetic acid or the hydrochloric acid series.

Because of its lethal activity at comparatively high pH values, the toxicity of acetic acid for various microorganisms is not confined to the hydrogen-ion concentration alone but also seems to be a function of the undissociated acetic acid molecule.

Grateful acknowledgment is made to Dr. L. A. Bradley and Dr. J. E. Fuller of the Department of Bacteriology for their cooperation in this investigation.

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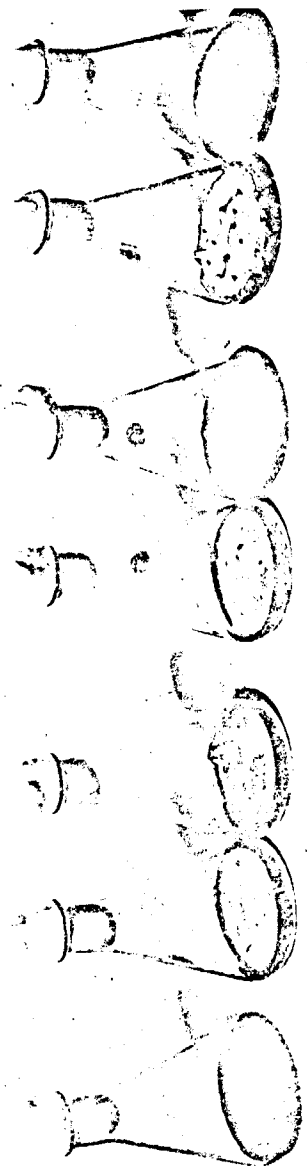
PLATE 1

COMPARATIVE EFFECTS OF ACETIC, HYDROCHLORIC, AND LACTIC ACIDS ON THE GROWTH OF ASPERGILLUS NIGER

Left to right:	Per cent acidity
Control	0.03
Acetic acid	0.27
Acetic acid	0.21
Hydrochloric acid	0.30
Hydrochloric acid	0.12
Lactic acid	3.48
Lactic acid	1.80



(A. S. Levine and C. R. Fellers: Acetic Acid and Food Spoilage)

Russian Translation

Some Problems Concerning the Clinical Aspects and Therapy of Severe Acetic Acid Poisoning

V.I. Lysenko (From the Center for the Prevention of Terminal States of the Frunze Medical First-Aid Station (Chief Physician: V.V. Lezhenkin) and the Second Hospital Surgery Clinic (Director: Prof. M.E. Fridman) based on the Combined Clinical Hospital No. 2 (Chief Physician: K.S. Nigmatullin, Honored Physician of the Kirgiz Republic).

Source: Sov. Zdravookhr. Kirg. No. 1, pp. 52-54, 1967.

Cases of poisoning by acids and alkalis are severe diseases often having a fatal outcome. According to I.V. Grigor'ev, the lethality in such cases is equal to 50%, and according to G.G. Zakharova, 21.6% (1957). Among common household poisoning by acids and alkalis, cases of acetic acid poisoning play a prominent role. Acetic acid seldom causes cicatricial complications, but results in a high lethality (G.G. Zakharov, 1957; P.L. Sukhinin, V.S. Tinkitnik, et al., 1964).

In the literature available to us, we were able to find only isolated reports on the treatment of small groups of patients suffering from acetic acid poisoning by means of blood exchange transfusion. O.S. Glozman and A.P. Kasatkina were the first ones to recommend this type of treatment in different types of intoxications caused by external agents. In their opinion, exchange transfusion can be used not only for disintoxication purposes but also in order to eliminate from the organism erythrocyte

hemolysis products; it also has a definite diuretic effect. O.S. Glozman and A.P. Kasatkina believe that the amount of blood necessary for the complete substitution of the recipient's blood by the donor's blood is 2 times greater than the total amount of blood present in the organism of the recipient; however, if the amount of donor blood available is small, it is better to perform exchange at a lower level than no exchange at all. In order to reduce the amount of blood used, V. Serafimov-Dimitrov (1963) has developed an experimental method for using a blood-dextran solution (blood diluted with an equal volume of a 6% dextran solution) in exchange transfusion procedures; in the course of exchange transfusions using a blood-dextran solution, he did not observe any noticeable shifts in the main functions of the organism, and only one experimental animal (out of 81) died during this procedure.

We have observed 90 patients admitted to the Center for the Prevention of Terminal States as a result of severe acetic acid poisoning. These patients belonged to different age groups; however, we found almost no cases of severe poisoning among children.

In regard to the outcome of cases of severe acetic acid poisoning and the method of treatment used by us, all patients under our observation were arbitrarily divided into 3 groups.

The first group included patients in which macroscopic hematuria, moderate amounts of protein in the urine, leucocytosis and accelerated erythrocyte sedimentation reactions were observed. In these patients, clearly expressed burns of the oral cavity and sometimes vomiting were also observed; however, no hemodynamic and respiratory disorders were noted, no blood was found in washing waters and in the stool, and no

clearly expressed hemolysis symptoms. This group included 19 patients, and not one of them died. Treatment of this category of patients included the use of analgetics, atropine, antihistamine preparations, antibiotics, copious gastric lavage, oxygen, intravenous infusions of low-molecular solutions, such as in isotonic sodium chloride solution, a 5% glucose solution, a 4% soda solution, novocaine administration, vegetable oil.

The second group included all cases of severe acetic acid poisoning. These patients exhibited a severe (serious) general condition, shock symptoms, vomiting (often with copious blood admixture), extensive burns of the alimentary tract, frequently hemodynamic and respiratory disorders, significant functional disorders of the kidneys, and a high hemolysis of erythrocytes. Treatment of these patients included, in addition to the measures mentioned above, also the use of cardiac and vasoconstrictive drugs, hormone preparations and paranephral blocks. Exchange transfusion played an important role in the treatment of these patients. Then, based on the assumption that the blood being transfused to the patient may possibly undergo a partial hemolysis, we started to practice bloodletting with simultaneous dextran transfusion, and then, in some cases, we switched to the usual exchange transfusion procedure.

Since we wanted to check our assumption on the possible hemolysis of donor erythrocytes during blood transfusion to the person suffering from acetic acid poisoning, we performed some special experimental studies, which did not confirm, however, the assumption made above.

Another rather important consideration favoring the use of dextran as a substitute for the recipient's blood is the fact that a sufficient amount of blood is not always available under the conditions found in

practice. By using dextran along with blood, we are able to perform the maximum possible transfusion by using a comparatively small amount of blood.

We have performed 15 regular exchange transfusions and 30 exchange transfusions with dextran in patients with severe acetic acid poisoning. The blood was obtained from the radial artery, in which an incision was made, and a microirrigator was inserted into the lumen of the vein. Transfusion of dextran and blood was done intravenously (by venesection, less frequently by venipuncture) or by the intraarterial route (in case of severe hemodynamic disorders).

The amount of blood extracted was equal to the total amount of blood and dextran transfused to the patient. The procedure was as follows: up to 2800 ml blood was taken from the artery, and simultaneously the same amount of dextran was transfused intravenously; then, in some cases, a regular blood exchange transfusion (up to 2000 ml) was performed.

According to our observations, the substitution of the recipient's blood by dextran (exchange transfusion) does not cause any kind of severe shifts in the organism. In most cases the condition of the patient improved after transfusion, acrocyanosis disappeared, the oxyhemoglobin content of the arterial network of capillaries increased, and there was a decrease in the ictericity (jaundice) of the sclerae and skin. In all cases, the procedure had a considerable diuretic effect, and was followed by a significant decrease of the hematocrit (down to 24-26), which, however, did not affect the general condition of the patient.

No noticeable change in the osmotic resistance of erythrocytes was noted after substitution of dextran for the recipient's blood. The transfusion procedure also did not affect the plasma content of sodium and

calcium, but a regular decrease of the potassium content was noted. In view of this fact, it is necessary to introduce, during the transfusion, an amount of potassium solution sufficient to compensate for this decrease. After the transfusion, a noticeable decrease of the total plasma protein content was noted, which gradually returned to its normal value. In some cases, a 20-30% reduction in the amount of erythrocytes and hemoglobin was noted, and also a sharp acceleration of the erythrocyte sedimentation reaction, which reached a normal value at the end of the first and the beginning of the second week. No noticeable shifts in the organism were noted in patients which we had the occasion to observe long after the transfusion.

The third group included patients admitted to the hospital in an extremely serious, often hopeless, condition and who died within 20 minutes to several hours after being admitted. This group included 15 patients.

Table

Percentage of fatal cases depending upon the gravity of the condition and methods of treatment

	Средней тяжести			Тяжелые			Крайне тяжелые		
	к-во	умерло	% летальности	к-во	умерло	% летальности	к-во	умерло	% летальности
Без операции замещения крови	19	—	—	20	11	55	6	6	100
Операция замещения крови	—	—	—	15	7	46,6	—	—	—
Операция замещения крови на полиглюкин	—	—	—	21	7	33,3	9	9	10.

1. Moderately grave
2. Grave
3. Extremely grave
4. Number
5. Died
6. Percent lethality
7. Without blood exchange transfusion
8. Blood exchange transfusion
9. Dextran exchange transfusion

Conclusions

1. Dextran exchange transfusion is an effective method for the treatment of patients suffering from acetic acid poisoning. With this method the general condition of the patients is improved, and the respiratory insufficiency and hemolysis of erythrocytes are reduced.

2. Substitution of dextran in the recipient's blood does not cause any pathological changes in the various functional systems of the organism, and therefore the use of this method is recommended in a number of cases.

В ПОМОЩЬ ПРАКТИЧЕСКОМУ ВРАЧУ

НЕКОТОРЫЕ ВОПРОСЫ КЛИНИКИ И ТЕРАПИИ ТЯЖЕЛЫХ ОТРАВЛЕНИЙ УКСУСНОЙ КИСЛОТОЙ

В. И. Лысенко

Из центра по борьбе с терминальными состояниями Фрунзенской станции скорой медицинской помощи (гл. врач — В. В. Леженкин) и клиники II госпитальной хирургии (зав.— проф. М. Е. Фридман) на базе объединенной клинической больницы № 2 (гл. врач — заслуженный врач республики К. С. Нигматуллин)

Отравления кислотами и щелочами являются тяжелыми заболеваниями, нередко приводящими к летальному исходу. И. В. Григорьев считает, что летальность при этом составляет 50%. По данным Г. Г. Захарова, она равняется 21,6% (1957). Среди бытовых отравлений кислотами и щелочами отравления уксусной кислотой занимают ведущее место. Уксусная кислота редко вызывает рубцовые осложнения, зато дает высокую летальность (Г. Г. Захаров, 1957; П. Л. Сухинин, В. С. Тиндитник и др., 1964).

В доступной литературе мы смогли встретить лишь единичные сообщения о лечении небольших групп больных с отравлениями уксусной кислотой операцией замещения крови. На целесообразность применения операции замещения крови при различного рода экзотоксикозах было впервые указано О. С. Глозманом и А. П. Касаткиной. Операция замещения крови, по их мнению, может применяться не только с дезинтоксикационной целью, но и с целью выведения из организма продуктов гемолиза эритроцитов; она обладает также несомненным диуретическим эффектом. О. С. Глозман и А. П. Касаткина считают, что для полного замещения крови реципиента кровью донора необходимо количество крови, в 2 раза превышающее общее количество крови, находящееся в организме реципиента, однако, если имеется мало донорской крови, то лучше сделать замещение меньшей степени, чем его не делать. С целью уменьшения количества используемой крови В. Серафимов-Димитров (1963) экспериментально разработал методику операции замещения крови на крове-декстрановый раствор (кровь, разбавленную равным объемом 6% декстранового раствора). Во время операции замещения крови на крове-декстрановый раствор он не наблюдал заметных сдвигов в основных функциях организма. Из 81 животного погибло только одно.

Мы наблюдали 90 больных, находившихся в центре по борьбе с терминальными состояниями по поводу тяжелого отравления уксусной кислотой. Больные принадлежали к различным возрастным группам, однако тяжелых отравлений среди детей мы почти не встречали.

Что касается исходов тяжелых отравлений уксусной кислотой и применявшегося нами лечения отравленных, то всех больных, находившихся под нашим наблюдением, мы условно разбили на три группы.

В первую группу мы включили больных, у которых наблюдалась макроскопическая гематурия, белок в моче в умеренных количествах, лейкоцитоз, ускоренное РОЭ. У этих больных были выраженные ожоги полости рта, иногда наблюдалась рвота. Но у них не отмечалось нарушений гемодинамики и дыхания, не было крови в промывных водах и стуле, не были выражены явления гемолиза. Больных этой группы

было 19. Из них не погиб ни один. Для лечения этой категории отравленных применялись анальгетики, атропин, антигистаминные препараты, антибиотики, обильное промывание желудка, кислород, внутривенные вливания мелкомолекулярных растворов: изотонического раствора хлористого натрия, 5% раствора глюкозы, 4% раствора соды, новокаин внутрь, растительное масло.

Ко второй группе мы отнесли все тяжелые случаи отравлений уксусной кислотой. У этих больных отмечалось тяжелое общее состояние, явления шока, рвота, зачастую с обильной примесью крови, обширные ожоги пищеварительного тракта, часто — нарушения гемодинамики и дыхания, значительные нарушения функции почек, высокий гемолиз эритроцитов. В лечении этих больных применялись кроме уже перечисленных мероприятий сердечные, сосудосуживающие средства, гормональные препараты, паранефральные блокады. Большое значение при лечении больных этой группы мы придавали операции замещения крови. Затем, исходя из предположения, что, возможно, кровь, переливаемая больному, частично гемолизируется, стали практиковать кровопускание с одновременным переливанием полиглюкина, а затем, в некоторых случаях — переходить на обычную операцию замещения крови.

Желая проверить наше предположение о возможном гемолизе эритроцитов донора при переливании крови отравленному уксусной кислотой, мы произвели специальные экспериментальные исследования, которые, однако, не подтвердили выставленного выше положения.

Другим, довольно веским, соображением в пользу применения замещения крови реципиента на полиглюкин является то, что в практических условиях не всегда имеется в наличии достаточное количество крови. Применяя наряду с кровью полиглюкин, мы можем, используя сравнительно небольшое количество крови, произвести как можно большее замещение.

Нами проведено всего 15 операций замещения крови и 30 операций замещения крови на полиглюкин у больных с тяжелыми отравлениями уксусной кислотой. Для извлечения крови мы пользовались лучевой артерией, которая надсекалась, и в просвет ее вводился микроирригатор. Переливание полиглюкина и крови производилось внутривенно (венесекция, реже венепункция) или внутриартериально (при тяжелом нарушении гемодинамики).

Количество извлеченной крови равнялось суммарному количеству крови и полиглюкина, перелитому больному. Операция производилась следующим образом: извлекалось из артерии до 2800 мл крови, одновременно внутривенно переливалось такое же количество полиглюкина, затем в некоторых случаях производилось замещение крови на кровь (до 2000 мл).

По нашим наблюдениям, операция замещения крови реципиента на полиглюкин не вызывает каких-либо тяжелых сдвигов в организме. После нее в большинстве случаев состояние больного улучшалось, исчезал акроцианоз, повышался оксигемоглобин артериальной сети капилляров, уменьшалось иктеричность склер и кожи. Во всех случаях операция оказывала значительный диуретический эффект. После операции наблюдалось значительное снижение гематокрита (до 24—26), что, однако, не сказывалось на общем состоянии больного.

После замещения крови реципиента на полиглюкин не отмечалось заметного изменения осмотической резистентности эритроцитов. Операция не сказывалась также на содержании в плазме натрия, кальция, однако мы обратили внимание на закономерное снижение калия. Этот факт наводит на мысль о необходимости введения во время операции раствора калия в количествах, компенсирующих это снижение. После

операции отмечалось заметное уменьшение общего белка плазмы, который постепенно приходил к норме. В некоторых случаях наблюдалось снижение количества эритроцитов и гемоглобина на 20—30%, а также резкое ускорение РОЭ, которая нормализовалась к концу первой — началу второй недели. У больных, которых нам приходилось наблюдать в отдаленные сроки после операции замещения крови, заметных сдвигов в организме не наблюдалось.

К третьей группе мы отнесли больных, поступивших в стационар в крайне тяжелом, зачастую безнадежном состоянии и погибших в сроки от 20 мин. до нескольких часов после поступления. Таких больных было 15.

Таблица

Процент смертельных исходов в зависимости от тяжести состояния и методов лечения

	Средней тяжести			Тяжелые			Крайне тяжелые		
	к-во	умерло	% летальности	к-во	умерло	% летальности	к-во	умерло	% летальности
Без операции замещения крови	19	—	—	20	11	55	6	6	100
Операция замещения крови	—	—	—	15	7	46,6	—	—	—
Операция замещения крови на полиглюкин	—	—	—	21	7	33,3	9	9	100

ВЫВОДЫ

1. Операция замещения крови на полиглюкин является эффективным методом лечения больных с отравлениями уксусной кислотой. Благодаря ей удается добиться улучшения общего состояния больного, уменьшения дыхательной недостаточности, снижения гемолиза эритроцитов.

2. Операция замещения крови реципиента на полиглюкин не вызывает патологических сдвигов в различных функциональных системах организма и поэтому применение ее в ряде случаев является вполне целесообразным.

Kirk-Othmer

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VOLUME 8

Electron Tube Materials
to
Ferrites

Mark, H. (Chairman), J. McKetta and D. Othmer
(Editorial Board)

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(ALSO RELATES TO FPC No. 0175)

ETHANOIC ACID

This article discusses ethanoic acid (acetic acid), and some closely related chemicals, as follows:

ACETIC ACID

Ethanoic acid (IUPAC), more commonly known as acetic acid (methanecarboxylic acid), CH_3COOH (abbreviated as AcOH), is a clear, colorless liquid with an acrid taste and pungent odor. As the acid of vinegar, acetic acid is as old as fermented liquors, which sour spontaneously, and which are historically recorded prior to 3000 BC. Today acetic acid is one of the most important industrial organic acids. It is produced mostly synthetically in volume exceeding a billion pounds per year. Acetic acid is most widely known in the form of vinegar, a dilute aqueous solution; it occurs both free and combined in the form of esters of various alcohols in many plants, and has been detected also in animal secretions (1–4). Major uses of acetic acid are in the manufacture of cellulose acetate fiber and plastics, ester solvents, dyes, metal salts, and many other chemicals. "Glacial" is a term applied to acetic acid of high purity (above 99%), which congeals to ice-like crystals at 58–60°F. Contact with concentrated solutions (50% or more) can cause severe skin burns and severe damage to the eyes, possibly resulting in total loss of sight. Exposed areas should be flushed immediately with ample quantities of water. Breathing of concentrated vapors is also harmful and swallowing may cause severe injury or death.

Physical and Chemical Properties

The physical properties of acetic acid are listed in Table 1. Freezing points and specific gravities of aqueous acetic acid solutions of varying concentrations are presented in Table 2, and acetic acid temperatures at selected vapor pressures are listed in Table 3. Figure 1 illustrates some physical properties of acetic acid–water solutions.

Acetic acid is miscible in all proportions with water, ethanol, and ether. It is an excellent solvent for organic compounds and is widely used as such in organic synthesis and in the preparation of acetates, acetone, acetic anhydride, etc. A dipole moment of zero for the unsymmetrical acetic acid structure has been explained by the formation of symmetric dimers via hydrogen bonding in which the dipole moments cancel. Highly dissociated ionic species do not occur in acetic acid solution because of the low dielectric constant of such a solution (10).

Acetic acid also exhibits relatively low basicity, or proton affinity, and as a solvent yields relatively small ionization constants for strong acids such as perchloric acid. Acetic acid serves as a differentiating solvent for strong acids such as perchloric, hydrobromic, sulfuric, hydrochloric, and nitric acids, which have nearly equal strength in aqueous solution, due to the leveling effect of water. Since acetonium ion, $CH_3C(OH)_2^+$, the strongest acid available in solution, is stronger than hydronium ion, acetic

Table 1. Physical Properties of Acetic Acid

melting point, °C	16.6
boiling point, at 760 mm, °C	118.8
density, d_4^{25}	1.0492
refractive index, n_D^{20}	1.37182 (5)
freezing point, see Table 2	
apparent specific gravity, at 20/20°C	1.0510 (6)
coefficient of cubical expansion, at 20°C	1.071×10^{-3}
critical pressure, atm	57.2 (7)
critical temperature, °C	321.6 (7)
dielectric constant	
2°C (solid)	4.1 (7)
20°C	6.15 (7)
electrical conductivity, at 25°C, mho/cm	1.12×10^{-8} (7)
ionization constant, at 25°C	1.753×10^{-5}
$-\log K$	4.76
magnetic susceptibility, cgs	-0.526×10^{-6} (7)
specific inductive capacity, at 18°C	9.7
surface tension, at 20°C, in air, dyn/cm	27.6 (7)
vapor density (air = 1)	2.07
viscosity, cP	
20°C	1.22 (9)
40°C	0.90 (9)
specific heat, at 0°C, cal/(g) (°C)	
liquid	0.468 (8)
solid	0.487 (8)
heat of combustion, kcal/mole	−209.4 (7)
heat of formation, kcal/mole	−116.2 (7)
heat of fusion, at 16.7°C, cal/g	44.7 (7)
heat of solution, at 18°C, kcal/mole	0.375 (7)
heat of vaporization, at 118.1°C, cal/g	96.8 (7)
autoignition temperature, °C	565
explosive limit, in air (lower limit), % by vol	4
flash point (Tag open-cup), °F	130

acid is very useful as a solvent in carrying out reactions that require stronger acids. Acetic acid, however, has a very strong leveling effect on bases and solvolyzes all strong bases to acetate ion, CH_3COO^-. This amphoteric behavior of acetic acid systems is demonstrated by zinc acetate which dissolves in acetic acid solutions of both hydrogen chloride and sodium acetate, although it is only very slightly soluble in acetic acid alone (11).

The characteristic carboxyl group of acetic and other fatty acids has been considered a resonance hybrid in order to explain an acid strength greater than that of water and of the comparable alcohols (12). Further substitution of electronegative atoms, such as chlorine, tends to increase acid constants by inductive effect along the carbon chain to the oxygen atom. For example, the acid constant, K, is 1.86×10^{-5} for acetic acid, 1.5×10^{-3} for chloroacetic acid, 5.0×10^{-2} for dichloroacetic acid, and 2×10^{-1} for trichloroacetic acid (13,14).

CHEMICAL REACTIONS

Since acetic acid may be treated as a resonance hybrid, in the formation of esters protonation is assumed to occur on the carbonyl oxygen by at least three different

Table 2. Freezing Points and Specific Gravities of Water Solutions in Acetic Acid at 15.5/15.5°C

Acetic acid, %	Freezing point, °C[a]	Specific gravity[b]
99.9	16.43	1.0558
99.8	16.24	1.0561
99.7	16.04	1.0564
99.6	15.84	1.0566
99.5	15.65	1.0569
99.4	15.47	1.0572
99.3	15.28	1.0574
99.2	15.10	1.0577
99.1	14.92	1.0580
99.0	14.74	1.0582
98.9	14.57	1.0584
98.8	14.40	1.0587
98.7	14.23	1.0589
98.6	14.06	1.0592
98.5	13.90	1.0594
98.4	13.74	1.0596
98.3	13.58	1.0599
98.2	13.43	1.0602
98.1	13.27	1.0604
98.0	13.12	1.0606

[a] Each 1% propionic acid depresses the freezing point by 0.485°C.

[b] 3.0% propionic acid depresses the specific gravity by 0.0019; 5% depresses it by 0.0032; and 6% depresses it by 0.0040.

Table 3. Acetic Acid Temperatures at Selected Vapor Pressures

Vapor pressure, mm Hg	Temperature, °C	Vapor pressure, mm Hg	Temperature, °C
1	solid	60	50.84
5	solid	100	62.19
10	17.11	200	79.12
20	29.16	400	98.10
40	42.44	760	117.86

mechanisms. The usual method of esterification involves the reaction of acetic acid with an alcohol in the presence of an inorganic acid as catalyst. For example, acid is refluxed with the alcohol in the presence of 5–10% concd sulfuric acid. Phosphoryl chloride has also been demonstrated as a good catalyst for the esterification reaction (see also Esterification). Esters of low molecular weight are fairly soluble in water, and all esters are soluble in most organic solvents (15).

The formation of acetate esters is undoubtedly the most important commercial use for acetic acid. Large quantities of ethyl acetate, butyl acetates, glycol diacetate, steroid acetates, and other alcohol esters of acetic acid are produced and consumed in the U.S. The recent literature includes references to acetic acid ester synthesis starting with acetylene to give vinyl acetate by using residual acetic acid; ethylene glycol esterification in the presence of ion exchange resins; and catalytic esterifications of methanol, ethanol, and butanol in the presence of a variety of catalysts. The discovery that olefins oxidized in acetic acid solvent yield unsaturated esters such as

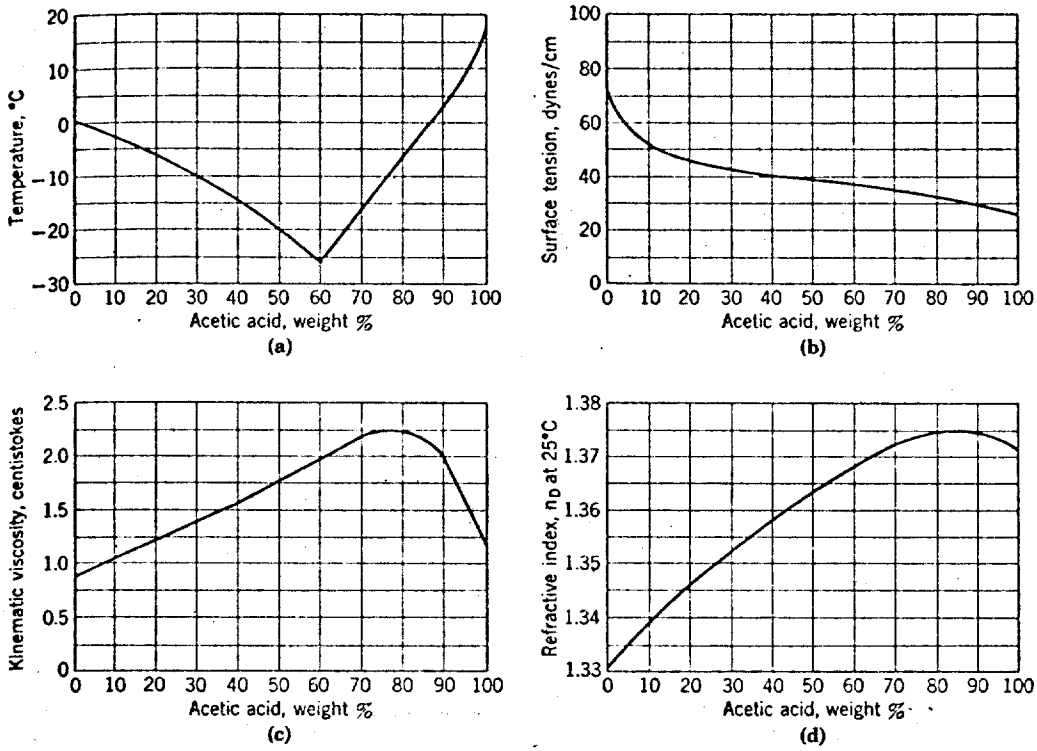

Fig. 1. Properties of acetic acid–water solutions. (a) Freezing points. (b) Surface tension at 25°C. (c) Kinematic viscosities at 25°C. (d) Refractive indexes at 25°C.

vinyl acetate and propenyl acetate, adds to the technical importance of acetic acid (16,17).

A yield of 97% of vinyl acetate based on converted ethylene is reported for the reaction in which palladium chloride is reduced by ethylene in solutions of acetic acid containing sodium acetate:

$$C_2H_4 + PdCl_2 + 2\,CH_3COONa \rightarrow CH_2{=}CH{-}OCOCH_3 + 2\,NaCl + Pd + CH_3COOH$$

Salts of acetic acid are formed directly by reaction with an alkali, or by the saponification or alkaline hydrolysis of an acetate ester. In equimolar proportions sodium acetate and acetic acid provide an excellent buffer solution. Sodium acetate, $CH_3COONa.3H_2O$, and potassium acetate CH_3COOK, are made by neutralization of acetic acid with the corresponding base. Acetates of many mono- and polyvalent metal ions have extensive commercial use. Lead acetate, $(CH_3COO)_2Pb.3H_2O$, one of the few water-soluble lead salts, is widely used in the manufacture of other lead compounds. Copper, zinc, and chromium acetates are commercially available inorganic salts. Copper ammonium acetate derived from either acetic acid or copper acetate is used to absorb butadiene from the butylenes. The industrial chemicals, white lead (basic lead carbonate) and Paris green (a copper acetate–copper arsenite complex), are also made from acetic acid (18).

Typical chemical reactions of acetic acid in addition to the formation of organic and inorganic salts include, halogenation, the formation of amide, nitrile, ketone, anhydride, and acid chloride, as well as acylation and solvolytic reactions.

Halogenation

$$CH_3COOH + Cl_2 \rightarrow CH_2ClCOOH + HCl$$

Substitution occurs at the methyl group and mono-, di-, and trichloroacetic acids are formed in turn when chlorine is passed into hot acetic acid

Amide formation

$$CH_3COOH + NH_3 \rightarrow CH_3CONH_2 + H_2O$$

Nitrile formation

$$CH_3COOH + NH_3 \rightarrow CH_3CN + 2\,H_2O$$

Ketene formation

$$CH_3COOH \xrightarrow{(600°C)} CH_2{=}CO + H_2O$$

Acid chloride formation

$$3\,CH_3COOH + PCl_3 \rightarrow 3\,CH_3COCl + P(OH)_3$$

Substitution of the hydroxyl group by a chlorine atom occurs.

Solvolytic reaction

$$4\,CH_3COOH + SO_2Cl_2 \rightarrow SO_2(OOCCH_3)_2 + 2\,CH_3C(OH)_2^+ + 2\,Cl^-$$

Metathetical reactions proceed smoothly in an anhydrous acetic acid medium despite its low dielectric constant. Solutions of, for example, heavy-metal salts, treated with hydrogen sulfide readily precipitate the appropriate sulfides. Silver nitrate solution gives precipitates with solutions of halides, cyanides, and thiocyanates. Sulfates that normally form hydrates are precipitated in anhydrous condition from a solution of almost any soluble metal salt by reaction with anhydrous sulfuric acid. Iron(III) thiocyanate complex is readily formed in acetic acid solution. Neutralization of metal salts with acids is also accomplished in acetic acid solutions; thus, sodium acetate is neutralized by hydrochloric acid with precipitation of sodium chloride.

Manufacture

The methods of producing acetic acid on a commercial scale have multiplied with the rapid expansion of the chemical industry, shifting in the last hundred years from natural fermentation processes to modern synthetic processes. Since the 1950s a growing proportion of the world's acetic acid has been produced by direct oxidation of petroleum fractions. Until then, most acetic acid was produced by the oxidation or dehydrogenation of acetaldehyde. Only minor amounts are still produced by wood distillation. Historically, the acetic acid industry began about 5000 years ago with the production of vinegar—the only acid reagent distinctly recognized by the ancients—by the fermentation of alcohol. It was not until the late nineteenth century that the distillation of wood in retorts was used to obtain acetic acid as well as methanol and acetone. The commercial availability of acetylene in the early 1900s led to a process for the production of acetaldehyde, which can be oxidized to acetic acid; this process was tried as early as 1905 but did not become economically important until 1916 (19,20).

ALCOHOL FERMENTATION PROCESSES

This ancient process employs the aerobic bacterial oxidation of alcohol to dilute acetic acid. Various acetic bacteria (*Acetobacter aceti*, including *A. curvum*, *A. orleanse*, and *A. schuezenbachii*) secrete an enzyme which promotes the oxidation of ethyl alcohol, possibly through acetaldehyde, to acetic acid. The two-stage fermentation reaction proceeds as follows:

$$C_2H_5OH + \tfrac{1}{2}O_2 \rightarrow CH_3CHO + H_2O$$

$$CH_3CHO + \tfrac{1}{2}O_2 \rightarrow CH_3COOH$$

The vinegar thus produced from cider, malt, or wine rarely has an acetic acid content greater than 5%; this rises to 12 or 14% if dilute alcohol is used. Modern commercial vinegar production employs the Orleans process or the quick vinegar process.

The quick vinegar process can be used for the commercial production of dilute acetic acid (8–10%). Blackstrap molasses is usually employed as an aqueous solution containing about 10% alcohol and 1% acetic acid. Large wooden tanks about 10 ft in diameter and 15 ft high filled with beechwood shavings are used as the "generators." A false bottom supports the packing and permits the introduction of air as well as the drawoff of the weak acetic acid. The alcohol solution is introduced at the top of the tank through a sparger and is trickled over the beechwood shavings. The weak acid is then recycled until the concentration reaches about 10%. Acid concentrations above approx 12%, however, tend to kill the bacteria. Phosphorus and nitrogen compounds are often supplied as nutrients to promote bacterial growth and thus yield a higher conversion of alcohol to acetic acid. The temperature of the fermenting mass is maintained at 30–35°C by controlling the air-flow rate. A 75–90% conversion of alcohol to acid yields 0.42–0.51 lb of acid per pound of reducing sugar in the molasses. Production by this method was formerly economically practical, up to a capacity of about 10 tons per day (21).

DESTRUCTIVE DISTILLATION OF HARDWOOD

From the destructive distillation of hardwoods, raw pyroligneous acid liquor is produced containing up to 7% acetic acid, 4% crude methanol and acetone, 9% tar and oil, and 80% water. The pyroligneous acid liquor is allowed to settle to separate the clear liquor from the tars. A preliminary distillation removes soluble tars and yields a boiled liquor. In a now obsolete process, after the acids in the distillate are neutralized with lime, methanol is distilled off, and the residue of calcium acetate solution is evaporated to dryness. Distillation of the calcium acetate with strong sulfuric acid and subsequent rectification produce acetic acid of glacial strength together with weaker fractions.

Direct Recovery Processes. Three major methods were worked out for recovering the acetic acid from pyroligneous liquor directly, without first forming calcium acetate, and then using sulfuric acid: (*1*) the extractive distillation process, or Suida system; (*2*) the cold liquid–liquid extraction process or Brewster system and its modifications; and (*3*) the azeotropic distillation process, or Othmer system.

Examination of Figure 2 will show that the vapor composition line for the system acetic acid–water is never far from the 45° line. Therefore, although the difference in boiling point is 18.8°C, separation by distillation to produce either component reason-

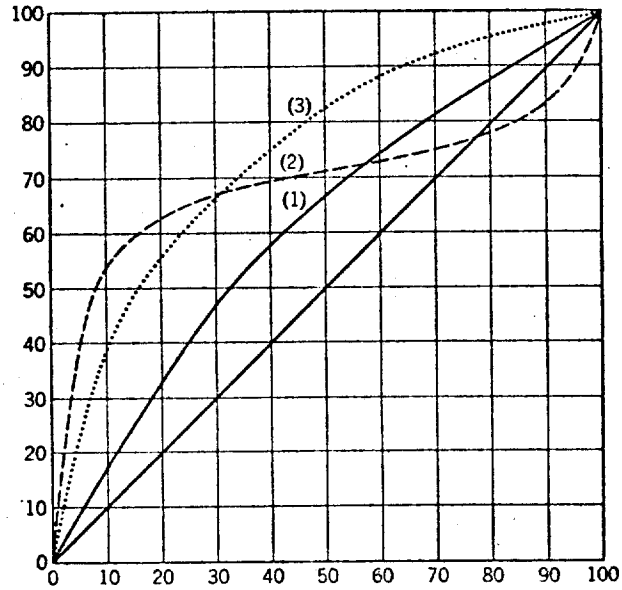

Fig. 2. Vapor–liquid composition curves: (1) acetic acid–water at 760 mm; (2) acetic acid–cyclohexane at 745 mm; (3) acetic acid–isopropyl ether at 742 mm. Value of y = mole % of more volatile component (water, cyclohexane, or isopropyl ether) in vapor phase; x = mole % of more volatile component in liquid phase.

ably pure would require a very large column, and a high reflux ratio and, consequently, a high steam consumption (see Distillation). The three processes described below represent three solutions to this problem. See also below under Acid concentration and recovery.

The Suida extractive distillation process passes the vapors from the distillation of settled pyroligneous acid through a countercurrent scrubbing column to dissolve the acid in a wood-oil tar fraction, while dilute methanol vapor is removed overhead to a recovery system. In a dehydrator water is removed from the acid–wood oil solution, which is then fed to a vacuum exhausting column to separate acid from oil. The oil is recycled and the acetic acid vapor is passed into the bottom of a rectifying column also operating under a vacuum and equipped with reflux condenser. Acid concentrated to 90–95% is withdrawn as liquid from the rectifier to vacuum receivers, and condensate from the top of the column is scrubbed with water to produce a weak 15% acetic acid. The Suida system, one of the first of the direct processes, has been used in Europe and in the United States; it does not yield anhydrous acid (22,23).

The cold-solvent extraction process in the Coahran modification of the Brewster system vaporizes acetic acid and methanol from the pyroligneous acid in a simple primary distillation, leaving soluble tar behind. About 85% methanol is removed overhead in a methanol column while the dealcoholized liquor is cooled and fed to a mechanical extraction column where about 98% of the acid is extracted by an ascending stream of cold ethyl ether (isopropyl ether and ethyl acetate have also been used as extracting agents). From the bottom of the extractor, the water layer (raffinate) is stripped of ether in an exhauster. Separation of the 2–3% acetic acid from the ether in the extract layer is accomplished by distillation in an evaporator and an ether column. Ether is recovered overhead, rectified, and reused, while crude acetic acid (70%) is discharged

from the bottom along with wood oils and tar. The crude acetic acid is treated with sodium dichromate as an oxidizing agent, and refined by either continuous or batch distillation to concentrations ranging from 90% to glacial (24–26).

The Othmer azeotropic distillation process pumps the settled pyroligneous acid to a demethanolizing column from which crude methanol is removed overhead, wood oil from a middle plate, and a 7–8% acetic acid solution from the base. This crude acid is fed into a preevaporator to distill off acid and water to an azeotropic dehydrating column. The preevaporator has a conical bottom for the removal of tars polymerized by the addition of small amounts of sulfuric acid. A withdrawing agent (either butyl acetate or ethylene dichloride) forms an azeotropic system with the water and acid vapor in the dehydrator. The withdrawing agent and the water distill off as a low-boiling azeotrope and then are condensed and separated into two phases: The top layer with withdrawing agent is returned to the dehydrating column as reflux wash, and the lower water layer goes to a stripping column to recover the small amount of the remaining withdrawing agent. Crude acid containing acetic, formic, propionic, and butyric acids and some tar is removed near the bottom of the dehydrator and is then rectified in one or two continuous columns from which acetic acid at concentrations of 99.5% or better is distilled. This process requires a minimum of equipment and is economical to operate with relatively small plants (27–31). (See also Vol. 2, pp. 850–853.)

SYNTHETIC METHODS

The major part of the world's production of acetic acid is made today by the synthetic rather than by the natural processes. The principal synthetic processes currently employed include (*1*) oxidation of acetaldehyde (qv); (*2*) direct oxidation of ethanol (qv); (*3*) hydrocarbon oxidation (qv); and (*4*) methanol–carbon monoxide process, as well as other miscellaneous methods.

Oxidation of Acetaldehyde. The production of acetic acid by acetaldehyde oxidation has an extensive patent literature going back more than fifty years. It has been in commercial operation since 1911 in Germany and 1920 in the U.S. Since the bulk of acetic acid in the world today is obtained from acetaldehyde, the acetaldehyde processes enjoy a leading position in any consideration of acetic acid manufacture. In many processes acetaldehyde is essentially an intermediate in the production of acetic acid, and this section of this article should be read in conjunction with the article Acetaldehyde in Volume 1, which describes four main routes to acetaldehyde: (a) the vapor-phase dehydrogenation or partial oxidation of ethyl alcohol; (b) the liquid-phase hydration of acetylene; (c) the high-temperature oxidation of saturated hydrocarbons; and (d) the liquid-phase oxidation of ethylene (32,33).

The continuous oxidation of acetaldehyde in the liquid phase is generally carried out by using air or oxygen in the presence of manganous acetate. The reaction mixture containing acetaldehyde diluted with crude acid and manganous acetate solution is circulated upward through the oxidation tower. Reaction conditions when air is used are 55–65°C at 70–75 psi (about 5 atm); and when oxygen is used, 70–80°C at a pressure sufficient to keep the acetaldehyde liquid. Oxygen is often diluted with 5% air to slow down the reaction and to avoid overoxidation which results in excessive quantities of by-products. The reaction mixture is drawn off the top of the oxidation tower and is distilled continuously in as many as three distillation columns. Crude acid is fed into the top of the distillation column and other volatile components are withdrawn overhead while a residue containing manganese acetate is removed at the

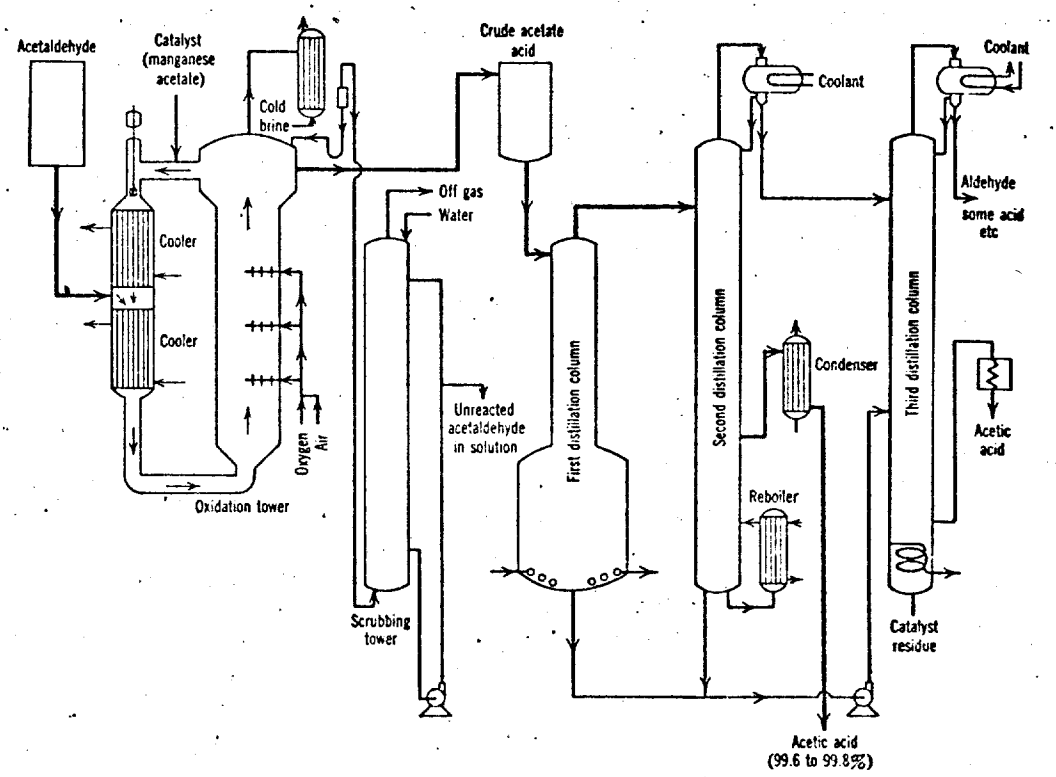

Fig. 3. Continuous oxidation of acetaldehyde in liquid phase.

bottom. A low-boiling forerun is taken off overhead in the second column and 99.6–99.8% pure acetic acid is then taken off above the reboiler. Bottoms from the first and the second columns and overhead from the second column go to a third column where acetic acid is taken off as a highly concentrated vapor, which is condensed and delivered to intermediate storage tanks (see Fig. 3).

The older batch process employed large aluminum-clad steel pressure vessels with extensive cooling coil surface to control the temperature. Oxygen was introduced through a sparger at the bottom to a mixture of acetaldehyde and crude acetic acid, and manganese acetate solution was added to destroy the explosive peroxyacetic acid formed at a temperature of 40°C. A 9–12 hr reaction time ended with sharp increase in pressure and simultaneous drop in temperature. The reaction mixture contained 93–94% acetic acid which was purified by distillation for a product yield of about 95% (34–36).

A modification of the acetaldehyde oxidation in which the catalyst is a mixture of copper and cobalt acetates is used for the simultaneous production of acetic acid and acetic anhydride (see below).

Direct Oxidation of Ethanol. This oxidation proceeds as a highly exothermic reaction of air and vaporized ethanol of high purity; the reaction takes place at 540–550°C and 25–30 psi. A very short reaction time, influenced by a silver-gauze catalyst, permits conversions of 50–55% per pass with product yields of 85–95%. Exhaust gases passing into a condenser and a scrubber absorb both the acetaldehyde and the unreacted alcohol into refrigerated water. Liquid taken from the bottom of the scrubber

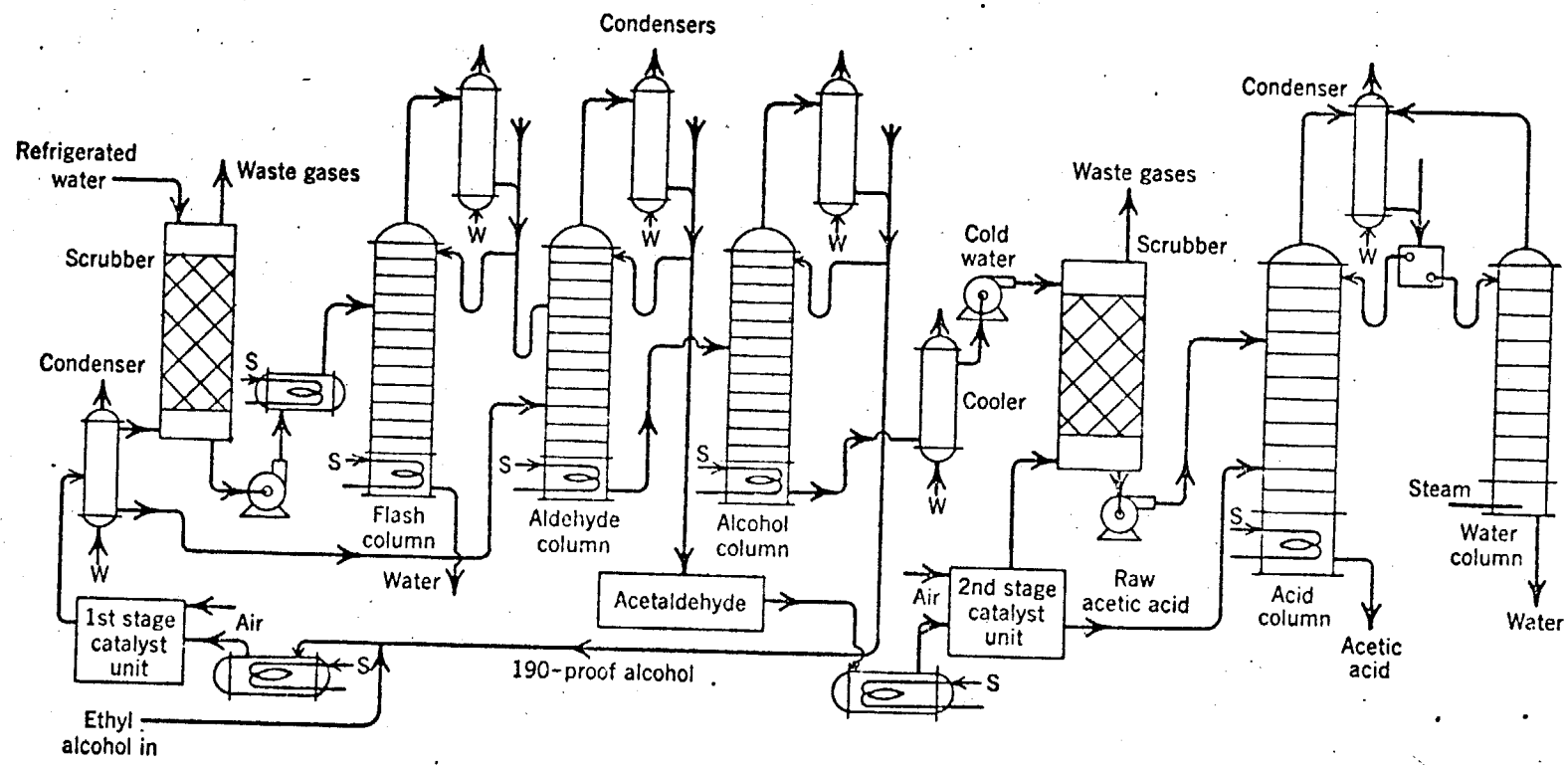

Fig. 4. Direct oxidation of ethanol.

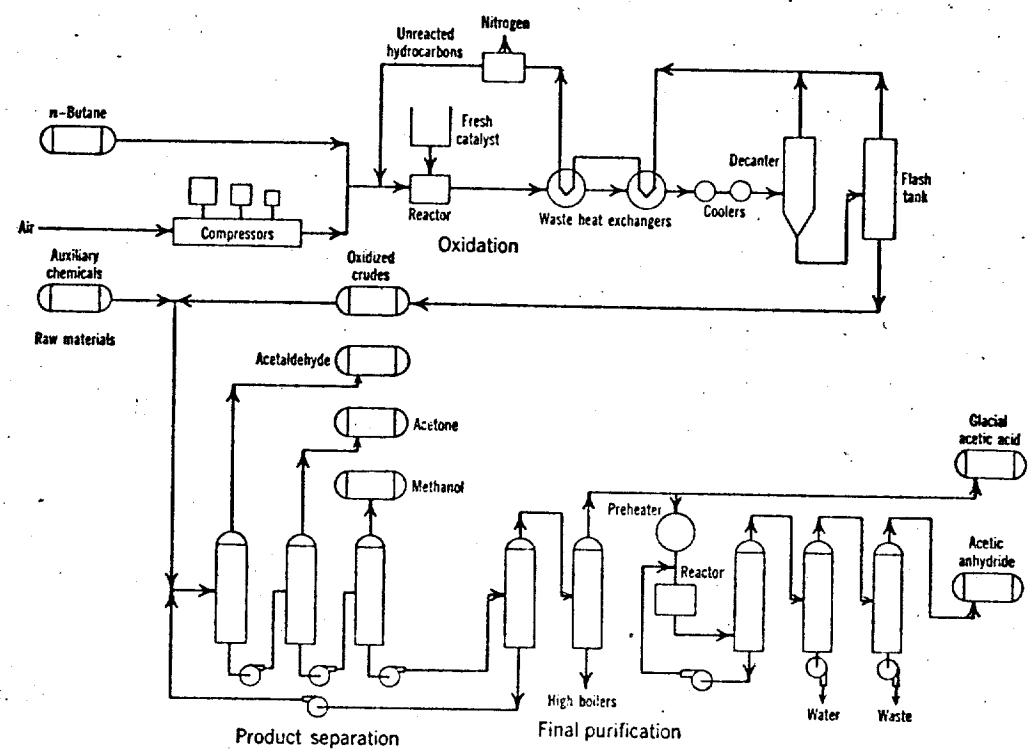

Fig. 5. Liquid-phase oxidation of hydrocarbons.

is a ternary mixture of water, acetaldehyde, and ethanol, the last two being recovered overhead after expansion in a flash column. Both the ternary mixture and the flash-column overhead go to an aldehyde column where acetaldehyde at 99% purity is recovered overhead by ammonia-refrigerated condensers. The bottoms are rectified in another column to recover 190° proof alcohol for recycling to the catalyst chamber (see Fig. 4).

Liquid-Phase Oxidation of Straight-Chain Hydrocarbons. In the presence of a suitable catalyst this oxidation yields acetic acid predominantly as the end product of the oxidation of intermediate oxidation products, such as esters and ketones. Continuous oxidation of lower aliphatic hydrocarbons, such as propane, *n*-butane, etc, is carried out at temperatures of 125–225°C and under pressures of 200–2000 psia using air, oxygen, or oxygen diluted with an inert gas as oxidizing agent. Acetic acid and other organic acids have been used as solvents for the hydrocarbons that undergo oxidation. Oxidation catalysts include a wide variety of organic acid metal salts. Salts of alkali metal and alkaline earth metals are used in different amounts as catalyst moderators to control reactions of the intermediate oxidation products. Compressed air and butane are fed to the reaction vessels at controlled rates, under conditions that ensure complete consumption of the oxygen. The overhead vapors consisting of water, unreacted hydrocarbon, and oxidation products are fed to a series of condensers that operate at 0–120°C, and include a battery of air-type heat exchangers. Nitrogen and other gases are separated; the nitrogen goes to drive turbines for the compressors used in the process, and the unreacted hydrocarbon is recycled to the oxidation reactors. The condensate is settled out into an upper hydrocarbon phase and a lower aqueous

phase. The hydrocarbon phase with dissolved oxidation products is also recycled to the reactor. The aqueous phase, consisting of water of reaction and water-soluble oxidation products, is treated through a separation train including acid concentration and recovery units. The lower-boiling compounds, esters, ketones, and alcohols, are separated from the acid and the water and returned to the reactor. The primary separation of acetic acid and water is made in a distillation tower which concentrates the acetic acid to near 100% in the base. The distillate contains about 15–20% acetic acid, which is recovered by extraction and distillation. The acid is distilled in additional columns to remove the residual water and the higher-boiling compounds (see Fig. 5).

Direct oxidation of light petroleum fractions obtained from refineries, largely butane, has been successfully adapted for commercial production of acetic acid in Europe since 1962 (37–48).

Methanol–Carbon Monoxide Synthesis. This synthesis has been employed by Du Pont and is described in the literature for the direct production of acetic acid in a number of processes involving phosphoric acid, metal oxides on activated carbon, and cobalt carbonyl as catalysts. A new process employed in the United States has been developed by Badische Anilin- & Soda-Fabrik; this process uses methanol and carbon monoxide derived from an acetylene unit based on natural gas. Carbon monoxide is first combined with hydrogen to produce the methanol which is reacted with additional carbon monoxide to yield acetic acid.

$$CH_3OH + CO \rightarrow CH_3COOH$$

The carbon monoxide gas is bubbled up through liquid methanol in a reactor at about 410°F and 7500 psig in the presence of a cobalt carbonyl catalyst. The reaction products in the form of gas and liquid mixtures are drawn off the top of the reactor. The gas stream is cooled to approx 100°F and is passed through a separator to remove unreacted alcohol for recycling; then it is fed to the bottom of a carbon dioxide absorption tower into the top of which cooled reactor liquid products are led. Carbon monoxide is drawn off the top of the absorber for recycling to the reactor. Liquid bottoms are decompressed to release absorbed carbon dioxide. The remaining liquid product contains acetic acid, water, methyl acetate, and catalyst which are separated by distillation in a recovery section. A theoretical yield of 72% acetic acid based on methanol is expected, making allowance for by-product methyl acetate production (49–56).

ACID CONCENTRATION AND RECOVERY

For more than a century the separation and recovery of acetic acid from its aqueous solutions has been of major economic importance to industry. The manufacture of cellulose acetate, aspirin, and the explosive RDX (cyclotrimethylenetrinitramine), as well as semichemical pulping, and other processes that use acetic acid as solvent or raw material, yield large quantities of spent solutions of dilute acid. The high reflux ratio necessary, which leads to high heat costs and excessive column diameter, has generally precluded the use of simple rectification of these dilute solutions. Three modern unit operations, now widely used for separation of industrial liquids, were developed largely for the recovery of acetic acid from spent processing liquors. These processes include (*1*) azeotropic distillation, in which another liquid increases the volatility of one component of two close-boiling liquids and thus separates them in one column; (*2*) liquid–liquid extraction, in which differential solubilities of

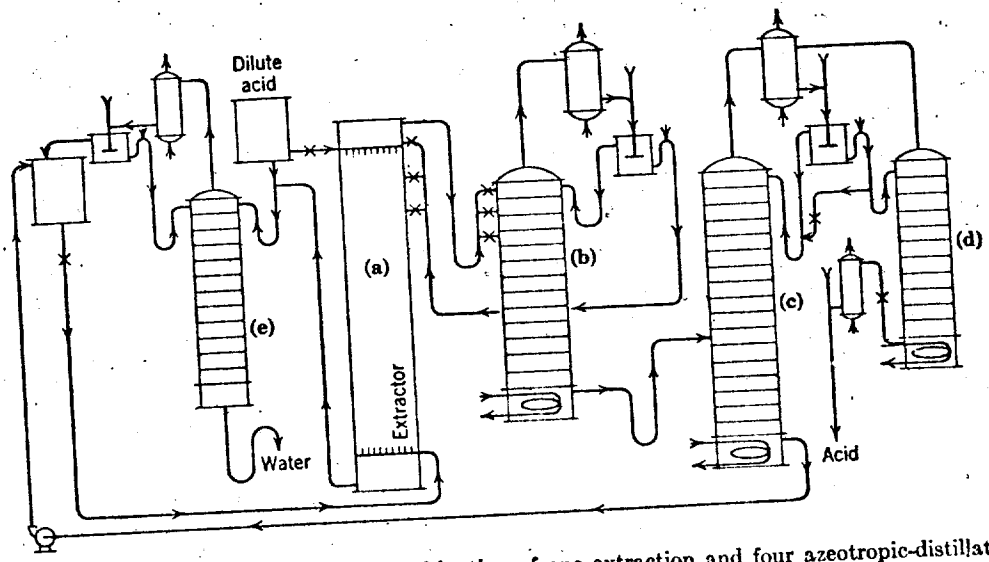

Fig. 6. Recovery of acetic acid by combination of one extraction and four azeotropic-distillation steps.

the two components in another liquid are used to wash one out of the mixture by countercurrent action; and (3) extractive distillation in which separation is effected by an agent or a solvent that is considerably less volatile than one of the feed components.

Many acetic acid recovery processes employ several of these methods consecutively for optimum heat economy. Extractions and extractive distillations are normally followed by further distillation operations, often azeotropic. A typical process (see Fig. 6) developed by Othmer uses an extraction and several azeotropic distillation steps in order to reduce the heat costs of concentrating acetic acid. A selective solvent separates acid from water in the extractor (a), and then distills the water overhead in a first azeotropic distillation column (b) with low reflux ratio. The condensed overhead, which contains a small amount of acid, is returned to the extractor at a point with the same acid strength. The bottom mixture is discharged to a second azeotropic distillation column (c) to separate acetic acid from the high-boiling solvent by the addition of another liquid which has much less solvent power for the acid than the solvent. Final exhaustion in an azeotropic distillation column (d) separates the second added liquid dissolved in the acid layer in a decanter, and the solvent dissolved in the water from the extractor is finally separated in a fourth column (e). This solvent and the solvent from the base of the second azeotropic column are recycled to the extractor (57–59).

Materials of Construction

Metals. Steady metallurgical advances tend to shift the emphasis in choice of materials for the handling of acetic acid. Constant improvement in the characteristics of stainless steel and aluminum alloys have led to increased use of these materials in this highly corrosive service. In the past, copper and copper alloys were used most extensively in equipment handling acetic acid, such as distilling columns for shell sections and plates. Copper is favored for its fabricability and low cost, but its corrosion rate is accelerated by high velocity and aeration, which occur in many of the modern

commercial processes. Copper may be used in continuous recovery processes where air can be excluded (60).

The impurities present in the acetic acid, their type and concentration, are extremely important factors affecting the life of various materials. Copper and its alloys, as well as Monel and nickel, have exhibited greater length of service in formic–acetic acid mixtures than in glacial acetic acid, although for austenitic stainless steel the reverse is the case. For hot mixtures of acetic acid containing over 2% formic acid, and at temperatures above 250°F, stainless steel may not be as satisfactory as copper alloys or Monel; for dilute acetic–formic acid solutions, however, molybdenum steels are preferred to copper and nickel alloys.

At room temperature aluminum has a corrosion rate of roughly 1 mil/yr in acetic acid of almost any concentration. However, at higher temperatures the rate tends to increase as the acid is diluted. A penetration of 10 mils/yr is reported for 1% acetic acid at 122°F, 7 mils for 60% acid, and 3 mils for 90% acid. Aluminum (Types 2S, 3S, 3003, and 61 ST) is considered suitable for use with the anhydrous acid and for infrequently used equipment, such as storage tanks. A major advantage of aluminum construction is that minute quantities of the metal do not discolor the acid product. When aluminum is used it should be shielded from electrolytic attack (particularly from contact with wet ground) by wrapping or by cathodic protection (61).

The austenitic stainless steels with their superior corrosion resistance are used increasingly in acetic acid service. Low carbon-, molybdenum-, and niobium-containing types (304, 304L, 310, 316, 316L, and 347) can be used for all concentrations of acetic acid at normal temperatures. Type 316 stainless steel is used for many hot acid services. Tests conducted by Eisenbrown and Barbis (62) under heat-transfer conditions in 56% acid indicated that, at various levels of chloride and sulfate ion content, Type 316 stainless steel had the best resistance of the alloys tested. Type 304L showed good resistance with 5.6 ppm chloride and 13.0 ppm sulfate, but at 20 and 25 ppm, respectively, high corrosion rates were obtained. Aluminum bronze and aluminum alloy No. 1100 had high corrosion rates at all levels of chloride and sulfate content. In acid mixtures with 56% acetic acid and 1% sulfuric acid, Type 310 stainless steel showed the best resistance; and with 5% sulfuric acid all alloys had high corrosion rates. The austenitic stainless steels perform well under oxidizing conditions generally because of the formation of an oxide surface film, whose formation has been shown to be a function of time. Reducing conditions as well as chloride ions tend to destroy the film and attack the metal underneath rapidly. High tensile stresses together with chloride-ion attack produce stress-corrosion cracking. Serious intergranular corrosion has also been reported in heat-affected zones adjacent to welds in austenitic stainless steels used for acetic acid service. Columns, bubble caps, stills, tanks, piping, pumps, and valves for acetic acid service are fabricated from stainless steel, preferably of welded and flanged construction. Type 304 stainless steel is used widely for storage tanks and tank cars.

The high alloys, with relatively large percentages of nickel, notably Hastelloy B and C, are suitable for service at all concentrations of acetic acid and at all temperatures. High cost and difficult fabrication, however, limit their use to critical applications where impurities are present. The Chlorimet alloys, which are also high in nickel, have good resistance to acetic acid and are most useful under oxidizing conditions. Inconel, another high alloy, is fairly resistant to the weak acid and is preferred to Monel for dilute, highly aerated solutions, however, it is not recommended for hot, concentrated solutions.

Nickel–copper alloys such as Monel have good resistance to unaerated solutions of acetic acid at all concentrations and at atmospheric temperatures, but the corrosion rate is greatly increased by aeration to about 8 mils/yr in aerated glacial acetic. Its use for hot aerated solutions is not recommended. Monel has been extensively used in pumps, reactors, heating coils, piping, and agitators for acetic acid service in unaerated solutions where sulfuric acid is present. The austenitic cast nickel–iron alloys, called Ni-Resist, have been used for unaerated solutions mostly at room temperature, and also for weak acid service at higher temperatures (63).

Cast iron and carbon steels are not recommended for acetic acid service. High-silicon irons, however, are used extensively in this service for valves, piping, pumps, towers, etc, independent of acid concentration, temperature, and degree of aeration. Brittleness, liability to damage from thermal shock, and costly fabrication methods are the major limiting factors to more widespread use of these materials.

Silver resists corrosive attack by acetic acid at all temperatures and all concentrations. It also has the advantage that its colorless organic compounds do not impair acid color. Where high mechanical strength is required, silver-clad materials or shrunk-fit linings are employed, especially for high-pressure or high-heat-transfer vessels (64).

Where good heat-transfer surfaces are required under severe corrosive conditions, tantalum has also found some application in this service. It shows excellent resistance to all concentrations of the acid at temperatures up to 175°C, and is mostly used in the fabrication of heaters and condensers.

Nonmetallic Materials. Plastic and ceramic materials are used in varying degrees for coatings, linings, and gasketing materials.

Stoneware is inert to acetic acid at all concentrations and temperatures, but has the disadvantages of relatively poor thermal conductivity and high initial cost. Stoneware and porcelain have been used for vessels, pumps, absorption towers, piping, etc.

Glass-lined steel is used in piping and other equipment handling acetic acid at all concentrations at atmospheric temperatures. As temperature increases, however, glass is subject to an increased rate of attack. Brittleness and susceptibility to damage from thermal shock are further drawbacks to the use of glass.

Carbon and graphite in their impervious forms are suitable for use up to the boiling point of acetic acid. Excellent heat-transfer properties have encouraged applications in heaters, evaporators, and condensers particularly. These materials have also found use in contact with acid mixtures such as acetic–formic and acetic–sulfuric acids.

Rubber and synthetic-rubber compounds display a broad spectrum of resistance to corrosion by acetic acid. In general, however, as temperatures and concentrations increase, these materials become less satisfactory, and to some extent may cause discoloration of the acid product. Hard rubber resists all concentrations and can be used at 120°F in contact with glacial acetic acid.

Plastics, although generally lacking in resistance to mechanical abuse, to most solvents, and to high temperatures, do not corrode electrolytically as metals do, and are not as greatly affected by changes in acidity, oxygen content, and impurities. Polytetrafluoroethylene (PTFE), which is used for valve components and valve packings, resists most chemicals and retains its properties up to 500°F. Polyethylene, however, is not suitable for acetic acid service since it permits the acid to diffuse through. Poly(vinylidene) chloride has been used for pipe lining in service up to 70°C where sufficient regard has been given to increased brittleness at higher temperatures.

Production and Use

Acetic acid exceeded the billion-pound annual production rate in 1963 to become one of the fastest growing of all chemicals—surpassed only by nitric acid and ammonia in rate of growth and total poundage consumed. Acetic acid showed a growth-rate pattern in the period of 1948 to 1963 of 6.2% per year or nearly one and two-third times faster than the Gross National Product during the same period. Acetic anhydride achieved an annual growth rate of 3.3% in the same fifteen-year period.

In the five-year period from 1959 to 1964, acetic acid production had grown from 672 million lb to 1112 million lb, amounting to about 75% of total available capacity. In the same five-year interval, sales or consumption of acetic acid grew by about 140%, averaging about 19% growth/yr. All of this growth is attributed to the new synthetic processes, since the production from pyroligneous distillation of wood declined from 20.8 million lb in 1950 to about 17.0 million lb in 1964 (see Table 4).

Table 4. U.S. Acetic Acid Production, 1950-1964, million lb

Year	Acetic acid		
	From wood	Synthetic	Total
1950	20.8	441.2	462.0
1951	19.4	454.0	473.4
1952	23.2	382.9	406.1
1953	20.4	477.7	498.1
1954	19.0	442.0	461.0
1955	22.8	524.0	547.2
1956	22.2	549.8	572.0
1957	19.8	524.4	544.2
1958	20.3	546.2	566.5
1959	23.5	648.7	672.2
1960	23.7	741.0	764.7
1961	19.9	764.0	783.9
1962	17.2	968.6	985.8
1963	17.0	1,028	1,045
1964	17.0	1,095	1,112

The largest single use for acetic acid is the production of acetic anhydride, which in turn is used to manufacture cellulose acetate, plasticizers, and pharmaceuticals. Cellulose acetate accounted for approx 45% of the acetic acid consumption in 1964. This use has grown almost by 40% during the five-year period from 1959 to 1964, averaging about 7% per year. In the same period, anhydride consumption for cellulose acetate production increased from 720 million lb in 1959 to 1 billion lb in 1964. Cellulose acetate finds greatest use in the production of acetate textile fibers, and is also employed in making lacquers, photographic film, transparent sheets, and thermoplastic molding compounds. See Acetate and triacetate fibers; Cellulose derivatives; Cellulose derivatives—plastics.

Vinyl acetate is the second-largest outlet for acetic acid and, from 1959 to 1964, has enjoyed an average annual growth of nearly 11% per year. A major raw material for vinyl plastics, vinyl acetate is also employed in the manufacture of latex paints, adhesives, and textile finishes. See Vinyl compounds and polymers.

The production of acetate esters consumes significant quantities of acetic acid. Methyl, ethyl, butyl, propyl, and amyl acetates find broad industrial use as solvents in the lacquer, plasticizer, and pharmaceutical fields. Ethyl acetate, for example, from 1948 to 1963, enjoyed an average annual growth rate of 3.9% per year and is a general solvent used in lacquer and plastics manufacture, as well as for flavorings, perfumes, and organic syntheses. Acetate esters accounted for roughly 16% of the acetic acid consumption in 1964. See Esters.

Chloroacetic acid (see p. 415) and acetate salts also constitute major outlets for acetic acid. Sodium acetate accounts for 60–65% of the consumption for all acetate salts including ammonium, copper, potassium, and zinc acetates. Sodium acetate finds application as a neutralizer and mordant in the manufacture of leather, textiles, and dyestuffs, as well as in photographic films.

Textile-finishing operations consume considerable quantities of acetic acid. Significant amounts are employed also in the production of nylon and of some acrylic fibers, as well as for dyestuffs and pigments.

The pharmaceutical industry continues to consume appreciable amounts of acetic acid in the production of vitamins, antibiotics, hormones, and similar products. Peroxyacetic acid and various rubber and photographic chemicals constitute a minor fraction of acetic acid usage (69–71).

Analysis and Specifications

The standard method of assay for glacial acetic acid (more than 99.4% acid) is the freezing point method and for dilute acid strengths titration with a standard alkali, such as sodium hydroxide, using phenolphthalein as the indicator. Water content in concentrated acetic acid is normally determined by the Karl Fischer iodine method.

The color of various basic acetates of the rare earths (eg, the blue of lanthanum acetate with iodine) gives a valuable means for unambiguous identification of acetic acid. Increasingly, the use of gas-liquid chromatography, however, has become the method of choice for both separation and identification of acetic acid (65,66).

Sensitive areas of application of acetic acid, such as in the drug, food, and solid-state electronics industries, have increased the importance of analytical procedures for trace impurities. Such impurities may include traces of formic acid, esters, aldehydes, sulfuric acid, sulfates, sulfurous acid, sulfites, chlorides, nitrates, arsenic, copper, lead, zinc, tin, iron, and other metals, depending on both process and equipment used.

Formic acid determination is made with mercuric chloride or lead tetraacetate. Formic acid reduces mercuric chloride to mercurous chloride, which is insoluble and can be determined gravimetrically. Lead tetraacetate (when used in excess) is reduced by formic acid, and the remaining reagent can be determined iodometrically. This method is preferred since it requires less time and is less subject to interferences. For esters, add alcoholic hydroxylamine hydrochloride and alcoholic potassium hydroxide until the solution is alkaline, warm until effervescence begins, and then cool; acidify with hydrochloric acid and add a drop of ferric chloride solution; purple color indicates esters. Aldehydes can be detected as bisulfite addition compounds, or by the addition of 2,4-dinitrophenylhydrazine in methanol solution with hydrochloric acid and neutralization with methanolic potassium hydroxide; a brownish-red color indicates aldehydes. The acid dichromate test may be used to detect formic acid, formaldehyde, and reducing substances such as occur in wood tars.

Sulfates and chlorides, diluted in water, are identified by the formation of precipitates with barium chloride and silver nitrate, respectively; sulfites are identified by the decolorization of starch iodide.

The following specifications indicate the chemical purity of acetic acid: purity, 99.85% by wt (min); specific gravity, at 20/20°C, 1.0505–1.0520; distillation range, 1°C, max; initial bp, 117.3°C, min; drying-up point, 118.3°C, max; freezing point, 16.35°C, min; color, Pt–Co units, max; water content, 0.15% by wt, max; formic acid, 0.05% by wt, max; acetaldehyde, 0.05% by wt, max; iron content, 1.0 ppm, max; heavy metals, 0.5 ppm, max; chlorides, sulfates, and sulfurous acid, 1.0 ppm, max; permanganate time (ACS test), 2 hr min (67,68).

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The Influence of Various Chemicals and Vitamin Deficiencies on the Excretion of Glucuronic Acid in the Rat

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Introduction

Certain aliphatic alcohols, aldehydes, ketones, terpenes, phenols, hydrocarbons, certain amines, aromatic acids, and many heterocyclic nitrogen compounds are detoxified by conjugation with glucuronic acid (1, 2, 3, 4). Many of the compounds thus detoxified are medicinal agents (5, 6, 7). It has been suggested that glucuronic acid might be used clinically to decrease the toxicity of sulfonamides (8) but the difficulty of preparing glucuronic acid in any degree of purity precludes the practical application of this procedure. It was therefore deemed advisable to seek a simpler chemical which would increase the formation of glucuronic acid in the body.

Procedure

Glucuronic acid (GA) content of the urine was determined by the use of naphthoresorcinol according to the technique of Maughan, *et al.* (9). As pointed out by Bueding (10) limited accuracy is achieved in applying any naphthoresorcinol method for GA. Some substances (glucose, etc.) inhibit the color development; others (mucic acid) tend to increase it. To circumvent this lack of specificity in method, the positive experiments were checked using the method of Quick (11) and the newer method of Bueding (12). It is felt by the present authors that the napthoresorcinol method can be safely applied to a dilute medium such as urine. This is in accord with the reports of other groups (13, 14, 15, 10). Meyer, *et al.* (15) stated, "None of the methods available gave accurate results for uronic acids in native fluids, with the possible exception of normal urine, since carbohydrates and other compounds containing carbonyl groups as well as proteins and their decomposition products interfered." In the experimental studies here reported, if, for example, mucic acid was being tested for its effect on GA excretion, a check was made using a solution containing an amount of the mucic acid equal

to the amount of GA determined. If at this concentration, the mucic acid gave a significant color, the series was discarded. Glucurone, prepared by the method of Williams (16) was used as the standard for all determinations.

All other chemicals unless specified were chemically pure reagent grade. The animals were housed in metabolism cages and urine samples collected under toluene for 24 hour periods after the oral administration of the chemical under test. Glass wool and special filtering devices prevented contamination of the urine samples.

TABLE I

The Effect of Various Compounds on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hr. period)

Compound	Dosage	Glucuronic acid excretion					
		80 mg./kg. Av. value for 15 sets of 12 rats each. Max. 95.2 mg./kg., Min. 73.4 mg./kg.					
Control	*g./kg.*	129.0	104.5				
Dihydroxyacetone	4	168.0	153.0	111.0	135.0		
Glycerine	4	117.0	98.0	119.0	140.0	127.0	91.0
Lactic Acid	2	109.0	60.0	44.0			
Ca Glycerophosphate	4	24	37	58	127	27	
Pyruvic Acid	2	55	105	81	118	58	
Acetic Acid	2	65	101	85			
Glycolic Acid	2	62	92	73	11	96	
Na Ethyl Oxalacetate	2	129	53	41			
Tartaric Acid	4	104	156				
Succinic Acid	4	231	178				
Malic Acid	4	69	83				
Fumaric Acid	6	86	99	80	98		
Citric Acid	4	132	125				
Saccharic Acid	4	94	105				
Glucurone	4	58					
Ca Phytate	4 ea.	106					
Glycerine + Malic Acid							
Adenylic Acid + Malic Acid	0.5 & 4	210					
Adenylic Acid	0.5	134	158				
Nucleic Acid	4.0	92	108				

Each value represents the average for 10 sets of 2 rats in each set.

Results

Table I presents the results obtained with carbon compounds. Included in this table are adenylic acid and nucleic acid.

Dihydroxyacetone, glycerine, and lactic, succinic, saccharic, malic, and adenylic acids increase GA excretion in the urine. Pyruvic acid decreased excretion of GA. It is believed that acetic acid, calcium glycerophosphate, glycolic acid, sodium ethyl oxalacetate, tartaric acid, and calcium phytate also decreased GA excretion, but variability of results prevents a final statement. Fumaric acid, citric acid, glucurone, and nucleic acid had little effect.

Of the amino acids tested, histidine, glycine, and alanine decreased GA excretion. Tyrosine and cysteine increased the excretion of GA. Cystine had no effect. Table II presents the results obtained. The results with ammonium chloride and sodium bicarbonate are included to show the effect of disturbance in alkaline acid balance.

TABLE II

The Effect of Various Amino Acids on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Compound	Dosage	Glucuronic acid excretion				
	g./kg.					
Histidine	2	56	76	54	74	
Cystine	4	94	63	94		
Glycine	8	73	35	60	48	51
Tyrosine	4	159*	130	151	190	141
Cysteine Hydrochloride	4	128	123			
Alanine	4	48	61			
Ammonium Chloride	2	46	61	55		
Sodium Bicarbonate	2	51	80	58	51	

* Rats receiving stock diet + 10% *l*-tyrosine.

Each value represents the average for 10 sets of 2 rats in each set.

With the exception of riboflavin deficiency, all the vitamin deficiency states resulted in a marked decrease in the excretion of glucuronic acid. Table III lists the results and includes a value showing the effect of 48 hour starvation on the excretion of the acid. All values presented are for rats taken in a stage of a given vitamin deficiency before they became moribund and while their food consumption was still adequate to prevent the intervention of the starvation factor.

With the sulfonamides, the results were variable. In acute experiments all tended to increased GA excretion excepting sulfapyridine. Sulfaguanidine was tried in a chronic experiment in which it constituted 2% of the diet. Under these conditions the rats after two months on the diet showed a marked drop in the excretion of the organic sugar acid.

TABLE III

The Effect of Various Vitamin Deficiencies on Glucuronic Acid Excretion

(Values expressed in mg./kg. of rat for a 24 hr. period)

Deficiency	Urinary glucuronic acid content					
Pantothenate	92	24	54			
B_1 Thiamine	20.9	45.8				
B_2 Riboflavin	114	39	77	107	103	127
D	21.9	33.8	48	105		
A	43.3	80	27			
E	2.3	17.7	15.2	44		
B_6 Pyridoxin	38	33				
Starved 48 hrs.	20					

Each value represents the average of 10 sets of 2 rats each.

TABLE IV

The Effect of Various Sulfonamides on Glucuronic Acid Excretion in the Rat

(Values in mg./kg. of rat for a 24 hour period)

Sulfonamide	Dosage	Added compound	Dosage	Glucuronic acid in urine				
	g./kg.		*g./kg.*					
Sulfanilamide	2				83	118	84	110
Sulfanilamide	2	Pyruvic Acid	4	76				
Sulfanilamide	1	Glucurone	2	80	198			
Sulfanilamide	2	Lactic Acid	4	75				
Sulfanilamide	2	Sodium Acetate	4	37				
Sulfanilamide	2	Malic Acid	4	94				
Sulfapyridine	4			59	31	28	34	
Na Sulfathiazole	1			210	134			
Sulfadiazine	6			97	60			
Sulfaguanidine	2% diet, 2 months			11.5	28.8			

Each value represents average for 10 sets of 2 rats each.

Tests conducted with sulfanilamide plus a compound known to produce no effect, positive or negative, on the excretion of GA showed that the sulfonamide tended to return all values to a more nearly normal figure.

Discussion

The objective of this work and of a companion effort (17) was to determine methods by means of which decreased acetylation and increased glucuronic acid formation could be brought about. The hope was that certain compounds might force the detoxication, particularly of sulfanilamide, away from acetylation and into combination with glucuronic acid. The literature contains ample evidence of the probability of this concept.

Our results *in vivo* in rats confirm those of Lipschitz and Bueding (18) *in vitro* using rat liver, in that dihydroxyacetone and lactic acid increased the formation of GA; but they differ in that we did not observe an increase following the administration of pyruvic acid, actually finding decreased excretion of GA. Quick (19) reported that lactic acid decreased GA formation in dogs, a point of dissimilarity with our findings in the rat. His result with glycolic acid is confirmed by our work. Another point of dissimilarity is in our observation that alanine decreased GA excretion, which would be expected if alanine were converted into pyruvic acid by oxidative deamination. Here, our results are in disagreement with both Lipschitz and Bueding (18) and Quick (19). Adeline (20) found that glycine, alanine, arginine, tyrosine, and histidine increased the output of glucuronic acid. In our experience alanine, glycine, and histidine decreased glucuronic acid formation, while tyrosine and cysteine increased its formation. It is probable that all seeming discrepancies observed are merely the result of dosage and species differences.

The sequence of potency with regard to stimulation of GA formation is in the order of decreasing effect: glycerine, dihydroxyacetone, and lactic acid. It is therefore suggested that the three carbon unit precursor sequence is from dihydroxyacetone phosphate to glycerophosphate and finally to phosphoglyceric aldehyde, which then condenses to a six carbon unit.

The phosphorylation of the precursors of GA was suggested by Lipschitz and Bueding (18). This work is corroborated by our observation that adenylic acid increased GA excretion. It is suggested that the high phosphate potential of adenylic acid aids in the phosphorylation of the three carbon unit precursor of GA. Further, this concept is strengthened by our observations that certain dicarboxylic acids, particularly succinic and malic, markedly increase the excretion of GA.

Kalckar (21) demonstrated in experiments with kidney extracts that the oxidation of dicarboxylic acids such as succinic, fumaric, and malic, gives rise to vigorous phosphorylation of various substrates. From the more recent work of Colowick, *et al.* (22) the conclusion is drawn that the generation of energy-rich phosphate originates with the dehydrogenation step from succinic to fumaric acid. It is therefore suggested that succinic and malic acids increase GA excretion in the rat by facilitating the phosphorylation of its three carbon unit precursor.

In riboflavin-deficient rats, the GA excretion is somewhat increased, this constituting the exception to the general rule of decreased excretion in various vitamin deficiency states. Riboflavin is involved in many enzyme systems, among these the dehydrogenation of malic acid in muscle, of lactic acid in muscle, etc. This might lead to increased concentrations of certain four carbon dicarboxylic acids and of certain three carbon units which lead to increased GA formation. The lactic acid oxidase system would be defective and prevent lactic acid, a compound stimulating GA production, from being oxidized to pyruvic acid, a compound inhibiting its formation.

It is much more difficult to explain the results seen with the other vitamin states. Liver pathology observed in rats in some deficiencies may explain defective GA synthesis.

Scudi and Robinson (23) have reported that sulfanilamide, in contrast to sulfapyridine and sulfathiazole, does not stimulate GA output. The work emphasizes the high percentage of sulfapyridine, some 40% of which is found in the form of a highly soluble glucuronide. This work and that of Weber, *et al.* (24) conclusively established the fact that sulfapyridine in the dog is followed by the appearance in the urine of a glucuronide of a hydroxy derivative of sulfapyridine. In our experiments, sulfapyridine clearly depressed GA excretion. It should be noted again that our experimental animals were rats, whereas Scudi and Robinson (23) and Weber, *et al.* (24) used dogs. Sulfanilamide, in our experience, increased to a slight degree the excretion of GA; sulfathiazole increased it to a marked degree; sulfadiazine had little if any effect; and sulfaguanidine fed chronically resulted in a profound decrease.

Summary

Dihydroxyacetone, glycerine, lactic acid, succinic acid, malic acid, and adenylic acid markedly increase glucuronic acid excretion in the rat. Of the amino acids tested, only tyrosine and cysteine had a positive effect. With the exception of riboflavin deficiency in which there is an increase in glucuronic acid excretion, all the other deficiency states showed decreased excretion of this acid. The effect of sulfonamides on the excretion of glucuronic acid by the rat was studied.

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REPORT ON ACETIC AND PROPIONIC ACID MOLD INHIBITORS IN BREAD

By Lewis B. McRoberts (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco, California), *Associate Referee*

In the previous reports on this subject (1—3, 4, 6, and 7), two methods were described for the determination of acetic and propionic acids in bread. The main purpose has been to provide means for the detection and estimation of propionates added as mold inhibitors. While acetic acid is normal in bread, the presence of excessive amounts would be indicative of the addition of vinegar or of chemicals such as sodium diacetate.

Following the adoption of the method described in the 1950 report (5, 6), the Referee on cereal products recommended that additional collaborative studies be made with breads other than white bread; milk bread and whole wheat bread with added propionate and sodium diacetate were suggested. Attention was also called to the possible presence of butyric acid which might interfere with the chromatographic separation of propionic acid. He also referred to the fact that lactic acid may be used in canned bread—primarily intended for Armed Services rations—and asked that experiments be conducted to determine any interferences from this source.

This report covers the requested collaborative study of the method (6) for the determination of propionic and acetic acids in milk bread and whole wheat bread. Results of the seven collaborators are reported and are found to be in good agreement. These data substantiate findings of previous studies on white bread. While there was some indication of trace amount of acids higher than propionic acid, the presence of butyric acid was not confirmed (3). To determine if interferences are caused by butyric

[2] For report of Subcommittee D and action of the Association, see *This Journal*, 36, 61 (1953).

or lactic acids, the Associate Referee conducted experiments in which mixtures of formic, acetic, propionic, butyric, and lactic acids were added to authentic control bread. There was no evidence of interference from lactic acid. Butyric acid interferes in the efficient separation of butyric and propionic acids where the concentration of propionic acid approaches 20 mg (about 1.6 times the usual amount of propionic acid in baked bread containing added propionate) in the 5 gram silicic acid chromatographic column. Under these conditions, the threshold volume for propionic acid is within a few ml of the preceding butyric acid. Where propionic acid is present in the amount indicated and where there are indications of butyric or other higher acids—i.e., visible bands below the propionic acid band—it is recommended that a longer tube with about a 10 gram charge of silicic acid be employed. A sufficient spread in threshold volumes between butyric and propionic acids is thus obtained to allow efficient separation.

COLLABORATIVE ANALYSES

The bread samples were prepared by the Referee on cereal products and transmitted to San Francisco by air express. The following information was supplied:

"Based on the weight of flour used (310–315 g per loaf) the formula was: 3 per cent shortening, 2 per cent salt, 2 per cent yeast, 3 per cent sugar, and 4 per cent dry skim milk (only in the wheat samples). Whole liquid milk was used for the milk breads. Calcium propionate and sodium diacetate were added in the amount of 0.63 g per loaf (0.20 per cent in the flour or 0.13 per cent in the finished bread). These breads were fermented, proofed, and baked by common procedure. Baking was at 425°F. for 32 minutes."

The weight loss during the one day shipping period was from 2.5 to 4.2 per cent. The breads used were:

(1) Milk Bread with propionate: 4 loaves; (2) Whole Wheat Bread, with propionate: 4 loaves; (3) Whole Wheat Bread, with sodium diacetate: 4 loaves. Two loaves of each kind were reserved for controls.

The loaves in each category were sliced and divided into two portions by taking alternate slices. One portion was prepared for analysis on the fresh basis and the other was air dried (6). One pint subdivisions of the prepared fresh breads were preserved with washed chloroform as directed in the method (6) and were submitted to six collaborators. They were instructed as follows:

"Each jar contains about 5 ml of washed chloroform and should be kept tightly closed before the initial opening for the analyses. Roll the closed jar to mix the sample thoroughly and break up any clumps of bread particles.

"Analyze the three samples for acetic and propionic acids as outlined in the method given in *This Journal*, 34, 284 (1951).

"It should be stressed that the threshold volume for propionic acid will vary

widely over the range of concentration found in bread and that identification of this acid by threshold volume alone must be based on previous determinations of several known concentrations similar to those described on page 289 (See footnote No. 5, pages 289 and 290.)

"There is some possibility that butyric acid may be present in amounts not large enough to provide a visible band on the chromatographic column. However, a titration of the forerun eluate that is greater than the blank titration is an indication of traces of higher acids. If time is available, the analyst is requested to determine if butyric acid is present by the formation of mercurous butyrate crystals [*This Journal*, 28, 644 (1945)].

"Analyze each sample in duplicate and report the following data:

(1) Blank titration, 20 ml CB 1 eluate
(2) Forerun titration (to first band) CB 1 eluate
(3) Number of bands observed
(4) Butyric acid (indicate if present)
(5) Propionic acid
  Threshold volume in ml
  Mg/100 g
(6) Acetic acid
  Threshold volume in ml
  Mg/100 g
(7) Formic acid
  Threshold volume in ml

"For best comparison of threshold volumes, the analysts are requested to change the mobile solvent from CB 1 to CB 10 at 40 ml of CB 1. This is about midway between the propionic and acetic thresholds."

The collaborative analyses of the three samples of fresh breads preserved with chloroform are tabulated in Tables 1, 2, and 3. Those of the Associate Referee are listed under Collaborator No. 1. The names of the others and corresponding numbers are given in the section devoted to collaborator comments.

In addition to the above-described analyses, the Associate Referee analyzed samples Nos. 1, 2, and 3 and control breads when they were first prepared fresh—without addition of chloroform, air dried portions of the same, and samples of the calcium propionate and sodium diacetate that were added previous to baking. Additional data were thus provided for comparison of methods of sample preparation. The results of these bread analyses are tabulated in Table 4.

The calcium propionate and sodium diacetate were analyzed by the following described procedures:

(a) *Calcium propionate.*—A weighed portion of 2.5 g was dissolved in H_2O, made to 250 ml, and an aliquot of 20 ml was made to 100 ml. Acetic acid (ca 1 ml 0.1 *N*), was then added to 10 ml of the final dilu. The mixt. was made alk. to phenolphthalein and evapd to dryness. Propionic acid was then separated on the silicic acid column as described in the bread procedure, using the added acetic acid as the following acid. Two bands were observed and identified as propionic and acetic acids by threshold volumes. A strong positive test was obtained for calcium in the ashed sample.

(b) *Sodium diacetate.*—A weighed portion of 2.5 g was dissolved in H_2O, made to 250 ml, and an aliquot of 30 ml made to 100 ml. Formic acid (ca 1 ml 0.1 *N*)

TABLE 1.—*Acetic and propionic acids in fresh authentic milk bread preserved with $CHCl_3$ (with added propionate)—Collaborative sample No. 1*

COLLABORATOR NO.	1	2	3	4	5	6	7	(Av.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.45	0.45	0.80	0.34	0.27	0.32	0.32	
No. bands observed	3	3	4	4	3	4	3	
Butyric acid	None	None	Present[c]	None[d]	None	None[d]	None	
Propionic acid								
Threshold vol. (ml CB1)[a]	18	17	15	19	15	14	15	16
mg/100 g	74.8	67.8	77.9	74.7	72.8	71.1	77.9	
mg/100 g	78.2	72.4	77.8	74.2	73.0	71.8	76.5	
mg/100 g (Av.)	76.5	70.1	77.9	74.5	72.9	71.5	77.2	74.4
Acetic acid								
Threshold vol. (ml CB1 + CB10)[b]	50	51	49	53	48	47	50	50
mg/100 g	34.2	36.8	31.3	36.4	34.4	44.7	29.6	
mg/100 g	35.2	35.8	34.9	30.6	35.2	44.8	31.3	
mg/100 g (Av.)	35.7	36.3	33.1	33.5	34.8	44.8	30.5	35.6
Formic acid								
Threshold vol. (ml CB1 + CB10)[b]	66	70	65	73	68	59	68	68

[a] CB1 (1% *n*-butyl alcohol in $CHCl_3$); [b] CB10 (10% *n*-butyl alcohol in $CHCl_3$); [c] Trace (1–2 mg/100 g) identified by threshold volume; [d] Slight band preceding propionic band—not identified.

TABLE 2.—*Acetic and propionic acids in authentic fresh whole wheat bread preserved with $CHCl_3$ (with added propionate)—Collaborative sample No. 2*

COLLABORATOR NO.	1	2	3	4	5	6	7	(AV.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.33	0.25	0.70	0.24	0.28	0.25	0.21	
No. bands observed	3	3	4	3	3	3	3	
Butyric acid	None	None	Present[c]	None	None	None[d]	None	
Propionic acid								
Threshold vol. (ml CB1)[a]	17	18	16	20	15	17	13	17
mg/100 g	79.3	76.1	82.7	76.7	76.0	78.5	82.2	
mg/100 g	79.7	69.2	82.9	77.2	74.5	76.3	81.9	
mg/100 g (Av.)	79.5	72.7	82.8	77.0	75.3	77.4	82.1	78.1
Acetic acid								
Threshold vol. (ml CB1+CB10)[b]	50	51	50	53	50	48	48	50
mg/100 g	38.5	42.8	39.9	37.1	43.2	38.4	38.9	
mg/100 g	38.2	44.7	42.7	37.4	44.6	39.4	36.6	
mg/100 g (Av.)	38.4	43.8	41.3	37.3	43.9	38.9	37.8	40.2
Formic acid								
(Threshold vol. (ml CB1+CB10)[b]	65	70	67	71	67	60	66	67

[a,b,c,d] See footnotes to Table 1.

TABLE 3.—*Acetic acid in fresh authentic whole wheat bread preserved with $CHCl_3$ (with added sodium diacetate)—Collaborative sample No. 3*

COLLAFORATOR NO.	1	2	3	4	5	6	7	(AV.)
Blank titration, ml 0.01 *N* $Ba(OH)_2$	0.05	0.05	0.10	0.03	0.05	0.05	0.05	
Forerun titration, ml 0.01 *N* $Ba(OH)_2$	0.60	0.33	0.42	0.61	0.35	0.52	0.34	
No. bands observed	2	3	3	2	2	3	2	
Butyric acid	None	None[b]	None[b]	None	None	None[b]	None	
Propionic acid	None	None[b]	None[b]	None	None	None[b]	None	
Acetic acid								
Threshold vol. (ml CB1+CB10)[a]	46	44	49	50	49	45	45	47
mg/100 g	133.0	129.5	118.2	116.9	120.0	114.4	119.2	
mg/100 g	127.0	132.0	121.3	110.2	119.0	116.7	125.7	
mg/100 g (Av.)	130.0	130.8	119.8	113.6	119.5	115.6	122.5	121.7
Formic acid								
Threshold vol. (ml CB1+CB10)[a]	64	66	65	73	69	59	68	66

[a] CB10 (10% *n*-butyl alcohol in $CHCl_3$); [b] Slight band preceding acetic band—not identified.

TABLE 4.—*Acetic and propionic acids in authentic breads (exp. 10) g—fresh basis)—Associate Referee analyses*

SAMPLE DESCRIPTION	FRESH BREAD				AIR DRIED BREAD (CALCD. FROM FRESH BASIS)					
	PROPIONIC ACID		ACETIC ACID		PROPIONIC ACID			ACETIC ACID		
	T. VOLS., ML	MG[a]	T. VOLS., ML	MG[a]	T. VOLS., ML	MG[a]	LOSS[b] PER CENT	T. VOLS., ML	MG[a]	LOSS[b] PER CENT
Milk Bread (Control)	None	None	50	84	None	None	—	50	26	26
Milk Bread (with added calcium propionate)	18	81	50	82	18	75		50	29	
	19	78	51	81	18	78		51	28	
(Av.)	19	80	51	82	18	77	15	51	29	15
Whole Wheat Bread (Control)	None	None	49	89	None	None	—	49	33	18
Whole Wheat Bread (with added calcium propionate)	18	84	50	87	18	79		50	32	
	17	84	49	90	17	80		51	32	
(Av.)	18	84	50	89	18	80	18	51	32	18
Whole Wheat Bread (with added sodium diacetate	None	None	48	127	None	None		48	101	
	None	None	45	127	None	None		48	101	
(Av.)	None	None	47	127	None	None	—	48	101	22

[a] Threshold volume. [b] Loss due to air drying. [c] Mg/100 g.

was then added to 10 ml of the final diln. The mixt. was made alk. to phenolphthalein and evapd to dryness. Acetic acid was then separated on the silicic acid column, making use of the added formic acid as the following acid. No propionic acid was detected. Two bands were observed and identified as acetic and formic acids by threshold volumes.

(c) As a check analysis, 10-ml aliquots of the final dilns of (a) calcium propionate and (b) sodium diacetate were combined with ca 1 ml of 0.1 *N* formic acid. Three bands were then observed and identified as propionic, acetic, and formic acids.

PROPIONATE AND DIACETATE ANALYSES

Calcium propionate

Propionic acid: chromatographic separation, 73.6 and 72.5%; average, 73.1%.
Propionic acid: calculated to $Ca(CH_3CH_2CO_2)_2 \cdot H_2O$, 100.7%.

Sodium diacetate

Free acidity: direct titration, 39.3 and 39.3%; average, 39.3% as acetic acid.
Total available acid: chromatographic separation, 78.8 and 78.5%; average 78.6% as acetic acid.
Combined acid: by difference, 39.3% as acetic acid; calculated as sodium acetate, 53.7% as acetic acid.
Total calculated: 93.0%.

BAKING LOSSES OF PROPIONIC AND ACETIC ACIDS

The following baking losses were calculated from the averages of collaborator analyses:

(1) *Milk bread with added propionate*

Propionic acid, calculated from formula:	94 mg/100 g
Propionic acid, determined:	74 mg/100 g
Propionic acid, baking loss:	21%

(2) *Whole wheat bread with added propionate*

Propionic acid, calculated from formula:	94 mg/100 g
Propionic acid, determined:	78 mg/100 g
Propionic acid, baking loss:	17%

(3) *Whole wheat bread with added sodium diacetate*

Acetic acid, calculated as added plus normal	144 mg/100 g
Acetic acid, determined:	122 mg/100 g
Acetic acid, baking loss:	15%

COLLABORATOR COMMENTS

V. E. Munsey (No. 2): "Mobile solvent was changed from CB 1 to CB 10 at 40 ml of CB 1. No definite band indications of higher acid were noted; a propionic band on No. 3 was faint. The forerun titration seems too small to be of significant interpretive value."

George E. Keppel (No. 3): "An attempt was made to identify the propionic acid and butyric acid fractions of the bread samples by crystal formation (*This Journal*, 28, 611 (1945)), but was not successful. Even with authentic acids, only with propionic and acetic acids did the crystals resemble those in the illustrations. The butyric acid fractions of the bread (samples 1 and 2) gave crystals similar to those obtained using *n*-butyric acid but neither resembled those illustrated.

"The threshold volume of the faint bands noted in samples 1 and 2 was 9–10

ml. This agrees approximately with the threshold volume of 8–10 ml found for butyric in a mixture of four acids. This was the same work done February, 1951, using the same batch of silica gel. Based on the threshold volume, I am therefore reporting butyric acid present in samples 1 and 2."

Louis C. Weiss (No. 4): "For sample No. 1, a narrow, barely visible band preceded the propionic acid. The amount of acid present in the band was so small that no attempt was made to identify it—in the case of sample No. 3, no bands were observed before the acetic acid zone.

"The technique for the preparation of the slurry suggested by Mitchell* was used throughout the work and was found to be very satisfactory. I prefer it to the grinding method.

"Bollinger's* method of releasing the acids from their salts and their subsequent transfer to the column was used. This too was found to be very satisfactory, as indicated by excellent recoveries on standardization runs.

"Some difficulty was experienced in the distillation procedure unless considerable care was exercised to prevent charring during the period before the charge in the distillation flask reached the boiling point."

Douglas D. Price (No. 5): "A negative test was obtained for butyrates in sample 2; other samples not tested."

Luther G. Ensminger (No. 6) ". . . No special difficulty was encountered in the procedure . . .

"In preparation of the column, I found that 0.5 ml (9 drops) of "RNH_4" indicator soln and 1 drop of 1 *N* NH_4OH added to the silicic acid gave a column easiest to follow the acid front. One ml of the indicator made the column too dark. 1.30 ml of H_2O was used to saturate the surfaces of the silicic acid particles. Three pounds pressure was used to drive the column.

"I used 25 ml beakers in place of the test tubes to complete evaporation of the distillate. Evaporation is faster, and by using a small glass stirring rod it is very easy to get all the residue quickly acidified and extracted with the CB 1 solvent."

Herman O. Fallscheer (No. 7): "No butyric acid band was observed on any of the runs."

DISCUSSION

The results obtained by the collaborators are considered to be in good agreement. Together with those of the previous report on white bread (6), they provide assurance that the method is adequate for general use in bread analysis in the detection and estimation of added propionates. Acetic acid has been definitely proved to be a normal constituent in all of the bread analyses. The amounts determined in control breads must be taken into account in judging whether acetic acid has been added.

The forerun titrations of the eluates to the thresholds of the first definite bands are always higher than a blank titration of about 25 ml of CB1 (1 per cent *n*-butanol in chloroform) that has passed through the column without addition of acids. This indicates the presence of traces of higher acids. We were especially interested in the possibility of detecting butyric acid in the milk bread. One collaborator identified butyric acid by threshold volume as present in trace amounts in both the milk bread and whole wheat bread. The others reported none or trace amounts too small to determine. No butyric acid was detected in the analysis of the control

* Collaborator Comments, *This Journal*, 34, 291 (1951).

breads. No definite mercurous butyrate crystals were obtained from the forerun eluates previous to propionic acid in samples Nos. 1 or 2 (Associate Referee analyses).

Data in addition to that provided in the previous report have been supplied on baking losses of propionic and acetic acids. Substantial losses are also obtained in the air drying of bread in preparation for analysis.

No appreciable differences are noted in comparing analyses of the fresh breads and the fresh breads preserved with chloroform (Table 4).

The following experiments were conducted to determine if small amounts of butyric or lactic acids might interfere in the determination of acetic or propionic acids by the method (6) used in this study (Table 5):

TABLE 5.—*Direct chromatographic separation of pure acid mixtures without distillation*

MIX NO.	LACTIC ACID	FORMIC ACID	ACETIC ACID	PROPIONIC ACID	BUTYRIC ACID	NO. OF BANDS
(1) Ml 0.01 *N* added	—	9.40	7.05	15.95	—	3
Ml 0.01 *N* recovered	—	—	6.96	15.28	—	
% recovered	—	—	99	98	—	
Threshold vol., ml[a]	—	65	50	16	—	
(2) Ml 0.01 *N* added	—	9.40	14.10	26.58	—	3
Ml 0.01 *N* recovered	—	—	14.14	25.92	—	
% recovered	—	—	103	98	—	
Threshold vol., ml[a]	—	65	47	13	—	
(3) Ml 0.01 *N* added	—	9.4	14.10	26.58	4.30	4
Ml 0.01 *N* recovered	—	—	14.20	27.29	2.49	
% recovered	—	—	101	103	58	
Threshold vol., ml	—	64	48	14	10	
(4) Ml 0.01 *N* added	—	9.4	7.05	—	4.30	3
Ml 0.01 *N* recovered	—	—	6.78	—	4.33	
% recovered	—	—	96	—	101	
Threshold vol., ml[a]	—	64	49	—	12	
(5) Ml 0.01 *N* added	—	9.4	7.05	5.32	4.30	4
Ml 0.01 *N* recovered	—	—	6.96	5.46	4.05	
% recovered	—	—	99	102	94	
Threshold vol., ml[a]	—	65	50	23	12	
(6) Ml 0.01 *N* added	4.30	9.40	7.05	5.32	4.30	5
Ml 0.01 *N* recovered	—	3.60	7.15	6.11	4.09	
% recovered	—	38	101	115	95	
Threshold vol., ml[a]	—	65	50	22	12	

[a] (Changed from "CB1" to "CB10" at 40 ml CB1).

The acids are eluted from the column in the order of butyric, propionic, acetic, formic, and lactic acids. Lactic is definitely above formic and moves very slowly even with the "CB10" mobile solvent. It could not

be used as a following acid in the determination of formic, for it seemed to merge with the formic band so that a clear-cut separation from formic acid was not possible.

With comparatively high amounts of propionic as compared to butyric acid (as in mixture 3) the threshold volume for propionic acid is within 4 ml of the preceding butyric acid. The recovery of butyric acid was definitely low and the separation of the two bands was not sharp. The separation of this same mixture was repeated with a longer column, making use of 10 g of silicic acid instead of the 5 g specified in the method. The following recoveries were then obtained: acetic acid, 101%; propionic acid, 93%; and butyric acid, 104%.

In general, the recoveries from the mixture described in Table 5 were considered good. Lactic and butyric acids had no effect on the acetic acid recovery. Where the propionic acid concentration approaches 20 mg, it may be necessary to increase the length of the column to afford efficient separation of propionic and butyric acids.

The following recovery experiments were made with addition of acid mixtures to the authentic whole wheat control bread. A chromatographic tube of 13 in. length and ½ in. internal diameter was used to afford sufficient space for 10 g of silicic acid. The usual mixture of 5 g silicic acid, 1 ml H_2O, 1 ml "RNHL" indicator and 1-2 drops 0.5 *N* NH_4OH was doubled for each ingredient in the column mixture (Table 6):

TABLE 6.—*Recovery of acid mixtures added to 10 g of air-dried authentic whole wheat control bread (complete method (6))*

MIX NO.	LACTIC ACID	FORMIC ACID	ACETIC ACID	PROPIONIC ACID	BUTYRIC ACID	BANDS NO.
(1) MI 0.01 *N* added	40.0	9.50	13.70	19.78	4.37	4
MI 0.01 *N* recovered	—	—	21.75	11.15	4.35	
% recovered	—	—	105[b]	103	100	
Threshold vol. ml[a]	—	85	61	39	23	
(2) MI 0.01 *N* added	80.0	9.50	13.70	21.56	4.37	4
MI 0.01 *N* recovered	—	—	21.25	21.40	4.25	
% recovered	—	—	101[b]	99	97	
Threshold vol. ml[a]	—	86	62	34	23	
(3) MI 0.1 *N* added	100	9.50	13.70	21.56	8.74	4
MI 0.01 *N* recovered	—	—	21.70	21.20	8.50	
% recovered	—	—	105[b]	98	97	
Threshold vol. ml[a]	—	87	62	36	22	
(4) MI 0.01 *N* added	200	9.50	13.70	43.12	4.37	4
MI 0.01 *N* recovered	—	—	22.75	41.50	4.00	
% recovered	—	—	113[b]	97	92	
Threshold vol. ml[a]	—	86	61	28	21	

[a] Changed from "CB1" to "CB10" at 20 ml "CB1."
[b] Called after correction for acetic acid detd in the bread (Table 4).

The lactic acid was apparently separated from the volatile acids during the steam distillation, for only four bands were noted on the columns. The amounts investigated varied from 36 to 180 mg or 0.36 to 1.8 per cent in the air dried bread.

The use of the longer column with 10 g of silicic acid gave a spread of about 15 ml between the threshold volumes of propionic and butyric acids, thereby affording a better separation of these acids.

CONCLUSIONS

The collaborative study conducted this year has demonstrated that the method is adequate for general use in bread analysis.

There was no definite proof of the presence of butyric acid in the authentic milk bread.

Lactic acid in amounts of 0.4 to 1.8 per cent does not interfere in the determination of acetic, propionic, or butyric acids.

Where the amount of propionic acid approaches 20 mg in the silicic acid column and where efficient separation from butyric acid is desired, a longer chromatographic tube and 10 g of silicic acid should be used.

ACKNOWLEDGMENT

The Associate Referee acknowledges with appreciation the assistance of the collaborators from the Food and Drug Administration whose names are listed under Collaborator Comments. He also wishes to thank the Referee on Cereal Products, V. E. Munsey, for his guidance and assistance throughout this project.

RECOMMENDATIONS*

It is recommended that the following directions be inserted in the method as it is now described in *This Journal*, 34, 284–296 (1951):

(1) Under III. APPARATUS, p. 287, change (*c*) to read: "chromatographic tubes ca 15×250 mm or ca 15×450 mm constricted at lower end to ca 4 mm i. d.

(2) Under CHROMATOGRAPHIC SEPARATION (*a*) *Preparation of partition column*, p. 288, add the following as a new paragraph at the end of (*a*):

Where the amount of propionic acid approaches 20 mg in the column and where a definite band is observed below the propionic acid band, the long chromatographic tube (450 mm) and ca 10 g of silicic acid should be employed. The amounts of water, indicator, and NH_4OH are then double those found applicable for 5 g silicic acid.

It is recommended that this Associate Refereeship be discontinued.

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COMPARISON OF LEUCINE WITH MEVALONATE AND ACETATE AS A PRECURSOR OF TISSUE AND SERUM CHOLESTEROL IN THE RAT

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ABSTRACT

Conversion of ^{3}H-leucine-^{14}C-acetate and ^{3}H-mevalonate-^{14}C-acetate mixtures to tissue and serum cholesterol was studied in normal and cholesterol fed rats in vitro and in vivo. Incorporation in vitro declines in the order 1) liver from mevalonate, 2) kidney, skeletal muscle and intestine from leucine, 3) liver from acetate. Calculated for whole tissue the largest amount of cholesterol was formed from leucine in skeletal muscle, followed by mevalonate and acetate in the liver. Cholesterol feeding inhibited hepatic cholesterol synthesis between acetate and mevalonate by 99 % in homogenate and 87 % in slices. Surprisingly, however, overall cholesterol synthesis from leucine was inhibited only by 63 % (homogenate) and 47 % (slices). Therefore the ^{14}C/^{3}H ratio in newly formed cholesterol decreased as if synthesis between acetate and hydroxymethyl glutarate (formed from leucine) had been inhibited by 96 % and 67 %. Thus, only 50—60 % of the leucine converted to cholesterol enters a feedback sensitive pathway, the rest is metabolized via a feedback-insensitive route.

After intraperitoneal injection of ^{14}C-acetate plus ^{3}H-mevalonate, the ^{14}C/^{3}H ratio in serum and liver cholesterol was reduced by a factor of 2—5 by cholesterol feeding. This indicates that cholesterol synthesis had been inhibited by 50—80 % between acetate and mevalonate. In agreement with the in vitro experiments, the ^{14}C/^{3}H ratio of serum and liver cholesterol after intraperitoneal injection of the ^{14}C-acetate/^{3}H-leucine mixture was significantly reduced by cholesterol feeding, but to a lesser extent than in the acetate-mevalonate experiment. Thus, dietary cholesterol inhibited cholesterol synthesis from leucine, although less effectively than from acetate probably owing to active cholesterogenesis from leucine via the feedback-insensitive route in the liver, and particularly in extrahepatic tissues.

INTRODUCTION

Leucine is metabolized through several intermediary steps to hydroxymethyl glutarate (HMG)* (14). Although this compound is a direct precursor of cholesterol (13, 24), and Bloch in 1946 (1) pointed out that deuterium-labelled leucine is converted to plasma cholesterol in the rat, no further attention has been paid to the role of leucine in the formation of serum cholesterol. Kabara (11, 12) showed that after intraperitoneal administration of ^{14}C-labelled leucine to normal and dystrophic mice, the specific activity was highest in intestinal, muscle and hepatic cholesterol. In man a significant amount of radioactive leucine appeared in serum cholesterol (18). Since this amino acid is metabolized at a higher rate outside than inside the liver (2, 21), it could be assumed that cholesterol synthesis from leucine takes place primarily outside the liver. Therefore, the significance of leucine as a precursor of serum and tissue cholesterol was in-

* Abbreviations used in this paper: HMG = hydroxymethyl glutarate; G-6-P = glucose-6-phosphate; NADP = nicotinamide adenine trinucleotide.

vestigated, in the rat, by measuring the incorporation of ^{3}H-leucine into cholesterol in vivo and in vitro. The results were compared with those obtained with ^{14}C-acetate and ^{3}H-mevalonate in normal and cholesterol-fed rats, using the double label technique (15). Some of the results have been presented earlier (19).

MATERIAL AND METHODS

Male Wistar rats (100—150 g) were kept for three days on a cholesterol-free diet (Fat-free Test Diet, Nutritional Biochemicals Co, Cleveland, Ohio) supplemented with 5 % of olive oil (w/w). The diet of half the rats was further supplemented with cholesterol (0.5 %) for an additional two or four days. The rats were then either killed for in vitro experiments or isotope mixtures were injected intraperitoneally. Serial blood samples for determination of cholesterol and radioactivity were taken from the tail by venous puncture under light ether anaesthesia.

Acetate-1-^{14}C, D,L-leucine-4,5-^{3}H and mevalonolactone-2-^{3}H (Radiochemical Centre, Amersham, England) were used as such with no further purification. The isotope mixtures used in vivo or in vitro were prepared in physiological saline as follows: mevalonate acetate 1 μCi 3 μCi, leucine-^{3}H acetate 4 μCi 1 μCi. The doses of isotopes administered intraperitoneally in these mixtures were 1 μCi of mevalonate, 12 μCi of leucine and 3 μCi of acetate.

Nicotinamide adenine trinucleotide was obtained from C. F. Boehringer & Soehne GmbH (Mannheim), glucose-6-phosphate as the barium salt from Sigma Chemical Co. (St. Louis, Mo.), L-leucine from F. Hoffman-La Roche A. G. (Basel) and DL-mevalonate from Fluka A. G. (Buchs SG).

Tissue preparations and incubation. Freshly removed liver, kidneys and intestine (the whole small intestine from pylorus to caecum) were homogenized in a three-fold volume of 0.1 M potassium phosphate buffer, pH 7.4, with a Potter-Elvejem-type homogenizer with a teflon pestle. The intestine was minced with scissors before homogenization. Skeletal muscle (quadriceps femoris) was homogenized in an Ultra-Turrax homogenizer (Janke-Kunkel). Homogenates were incubated as such or after removal of debris by centrifugation for 3 min at 600 g. Liver slices (0.5 mm thick) were prepared with a Stadie-Riggs microtome (Thomas, Philadelphia, Penn.) after cooling the piece of tissue in the phosphate buffer supplemented with 0.03 M nicotinamide, 0.001 M EDTA-2Na and 0.004 M $MgCl_2$.

Tissue homogenates (2 ml) or liver slices (0.5 g) were incubated (6) in glass-stoppered flasks at 37 C for 2 hr in 0.1 M potassium phosphate buffer, pH 7.4, with appropriate additions. The tubes were gassed with 95 % O_2 and 5 % CO_2 at 0 C before incubation in a Gallenkamp incubator (shaking rate 100 cycles/min).

Chemical analysis. Serum and incubation mixtures or liver pieces were saponified by heating the tubes at 60 C for 1 hr in a 10-fold volume of 1 N NaOH in 90 % ethanol. After cooling, water was added to give 60 % ethanol, and sterols were extracted with petroleum

TABLE 1

INCORPORATION OF ACETATE-1-^{14}C, LEUCINE-4,5-^{3}H AND MEVALONATE-2-^{3}H INTO CHOLESTEROL BY VARIOUS TISSUE HOMOGENATES IN THE RAT.

2.0 ml of 25 % tissue homogenate was incubated in medium containing 0.03 M nicotinamide, 0.001 M EDTA-2Na, 0.004 M $MgCl_2$, 0.0005 M NADP, 0.005 M G-6-P, 0.001 M mevalonate, 0.001 M leucine and 0.001 M acetate in 0.1 M potassium phosphate buffer, pH 7.4, at 37 C for 120 min. 2 μCi of either acetate-1-^{14}C and DL-leucine-4,5-^{3}H or acetate-1-^{14}C and mevalonate-2-^{3}H was added to incubation tubes, which were then blown with 95 % O_2 and 5 % CO_2 before incubation. Final volume 2.2 ml. Mean ± SD.

Tissue	No. of rats	Radioactivity incorporated into cholesterol, dpm/g of wet weight			dpm from acetate-^{14}C: leucine-^{3}H: mevalonate-^{3}H
		From acetate-^{14}C*)	From leucine-^{3}H	From mevalonate-^{3}H	
Liver	5	6082 ± 2260	2584 ± 335	98810 ± 32240	1:0.4:16
Intestine	5	35 ± 10	4516 ± 423	468 ± 129	1:130:13
Muscle	5	36 ± 16	7950 ± 546	326 ± 152	1:220:9
Kidney	5	140 ± 55	9149 ± 1530	3388 ± 688	1:65:24

* Calculated from the averages of the two experiments.

ether. Extracts were washed thrice with 50 % aqueous ethanol. In part of the petroleum ether extract total sterols were determined by the method of Hausen and Dam (9). The rest was evaporated and applied on a silica gel G chromatoplate (0.5 mm thick). The plate was developed in ethyl ether:heptane 1:1, throughout its length. The cholesterol fraction was eluted as decribed for faecal steroids (17). Part of the extract was used for determination of the mass with the colour reaction (9), the bulk being used for measurement of the radioactivity. Cholesterol isolated by this method from normal human serum, to which radioactive precursor mixtures had been added, did not contain any radioactivity. Thus the method of isolating cholesterol effectively removed unutilized radioactive precursors.

Radioactivity measurements were performed in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 574 (Packard Instruments Co, Inc. La Grange, Ill.) determining absolute activity levels (dpm) by means of an external standard. The scintillation liquid contained 5 g of 2,5-diphenyloxazole and 0.3 g 1,4-bis-2-(4-methyl-phenyloxazolyl)-benzene in 1 litre of toluene. The results are expressed as dpm/g of wet tissue or as dpm/mg of cholesterol.

RESULTS

Incorporation of ^{3}H-leucine, ^{3}H-mevalonate and ^{14}C-acetate into tissue cholesterol in vitro

The different tissue homogenates incubated with radioactive acetate, leucine and mevalonate, in the presence of their non-labelled carriers, incorporated these precursors into cholesterol at varying rates (Table 1). In the conditions employed, liver had the highest rate of conversion of acetate and mevalonate, while kidney and skeletal muscle had the highest rate of leucine conversion. The conversion of leucine to cholesterol in the muscle tissue was about 200-fold that of acetate and 25-fold that of mevalonate. Utilization of leucine for cholesterol synthesis was markedly higher than that of acetate and of mevalonate even in the intestine and kidney, while in the liver homogenate acetate and mevalonate were better precursors than leucine.

The incorporation of radioactivity into cholesterol in slices and homogenates of livers of normal and cholesterol-fed rats from the two precursor mixtures, ^{3}H-leucine plus ^{14}C-acetate and ^{3}H-mevalonate plus ^{14}C-acetate, are shown in Table 2. The conversion of mevalonate to cholesterol was not affected by cholesterol feeding, while that of acetate was reduced by 87 % in the slices and by 99 % in the homogenate. Thus, according to these experiments, 13 % (slices) and 1 % (homogenate) of hepatic cholesterol synthesis from acetate appears to proceed via a feedback-insensitive route. The ^{14}C ^{3}H ratio was high in the homogenate owing to high acetate incorporation. This ratio decreased about 8-fold and 80-fold in slices and homogenate respectively, in response to cholesterol feeding. This indicates that cholesterol synthesis between acetate and mevalonate was inhibited correspondingly by 99 % and 87 %.

Although leucine and acetate are both converted to HMG during sterol synthesis (13, 14, 24), the incorporation of leucine into cholesterol was inhibited only by about 47 % (slices) and 63 % (homogenate) in the liver of cholesterol-fed rats (Table 2). More pronounced inhibition of acetate incorporation (82 % and 99 %) resulted in a corresponding decrease in the ^{14}C ^{3}H ratio in cholesterol by 3-fold and 28-fold. This suggests that overall inhibition of cholesterol synthesis by dietary cholesterol between acetate and HMG (formed directly from leucine) was 96 % and 67 %. Thus, about 40—50 % of the leucine that is converted to cholesterol in the liver enters a metabolic pathway that is not inhibited by dietary cholesterol. The rest is metabolized through the route that is sensitive to feedback.

Incorporation of ^{3}H-leucine, ^{3}H-mevalonate and ^{14}C-acetate into serum cholesterol of control and cholesterol-fed rats

The significance of leucine as a precursor of serum cholesterol compared with that of mevalonate and acetate was tested in cholesterol-fed and control rats. In cholesterol-fed rats hepatic cholesterol synthesis is known to be suppressed (5, 7, 28). After intraperitoneal administration of a mixture of ^{3}H-mevalonate and ^{14}C-acetate the specific activity of serum ^{3}H-cholesterol was significantly reduced in

TABLE 2

EFFECT OF DIETARY CHOLESTEROL ON INCORPORATION OF ACETATE-1-^{14}C, LEUCINE-4,5-^{3}H AND MEVALONATE-2-^{3}H INTO LIVER CHOLESTEROL IN HOMOGENATES AND SLICES FROM RATS ON CHOLESTEROL-FREE DIET.

2.0 ml of 25 % liver homogenate (centrifuged for 3 min at 600 g to remove debris) or 0.5 g of liver slices (from the same rats as homogenate) was incubated in medium containing 0.03 M nicotinamide, 0.001 M EDTA-2Na, 0.004 M $MgCl_2$, 0.0005 M NADP, 0.005 M G-6-P and 0.001 M acetate in 0.1 M potassium phosphate buffer, pH 7.4, at 37 C for 120 min. 1 μCi of acetate-1-^{14}C and 4 μCi of DL-leucine-4,5^{3}H or 1 μCi of acetate-1-^{14}C and 1 μCi of mevalonate-2-^{3}H were added to the incubation tubes, which were then blown with 95 % O_2 and 5 % CO_2 before incubation. Final volume 2.2 ml. Mean ± SD of five rats.

Dietary cholesterol	Cholesterol, mg/g of wet liver	Incorporation of mevalonate-^{3}H and acetate-^{14}C into liver cholesterol, dpm/g wet weight				Incorporation of leucine-^{3}H and acetate-^{14}C into liver cholesterol, dpm/g wet weight			
		^{3}H	^{14}C	^{14}C ^{3}H×10^{-3}	Inhibition, % **	^{3}H	^{14}C	^{14}C ^{3}H×10^{-3}	Inhibition, % ****
				Homogenate					
None	2.76 ± 0.13	73075 ± 15080	14793 ± 4446	202.20	—	706 ± 114	17255 ± 10030	24200	—
0.5 %	6.17* ± 1.29	80350 ± 4770 (110)	206 ± 186 (1)	2.56	99	264* ± 96 (37)	232* ± 214 (1)	878	96
				Slices					
None	4.10 ± 0.70	125025 ± 36550	1827 ± 915	14.60	—	616 ± 62	2376 ± 809	3859	—
	7.26* ± 0.91	124700 ± 36260 (100)	233 ± 114 (13)	1.87	87	327* ± 24 (53)	418* ± 262 (18)	1279	67

* $P < 0.05$

** Inhibition of cholesterol synthesis between acetate and mevalonate, calculated from the change in ^{14}C/^{3}H.

*** Incorporation in cholesterol-fed rats relative to that in controls indicated by figures in parentheses (%).

**** Inhibition of cholesterol synthesis between acetate and hydroxymethyl glutarate (formed from leucine), calculated from the change in ^{14}C ^{3}H.

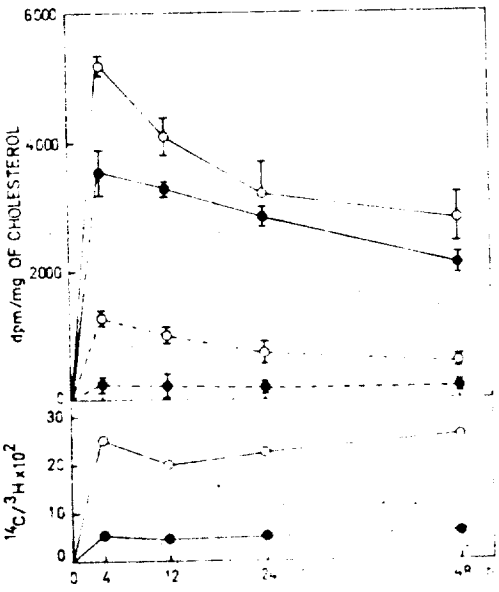

Fig. 1. Effect of cholesterol feeding on conversion of a ^{14}C-acetate-^{3}H-mevalonate mixture to serum cholesterol in rats on a fat-free diet. Each group consisted of five animals. Time curves of the ^{14}C ^{3}H ratio in serum total cholesterol in the lower panel. O controls, ● cholesterol-fed. — — ^{3}H-cholesterol, — ^{14}C-cholesterol.

cholesterol-fed rats (Fig. 1). Thus, either the conversion of mevalonate to cholesterol was inhibited, or the newly formed ^{3}H-cholesterol was diluted into an expanded cholesterol pool. Evidence for the latter was provided by the markedly increased cholesterol content of the serum and liver in cholesterol-fed rats (Table 3). In addition the amount of the label from ^{3}H-mevalonate recovered at 20 hr in the cholesterol of the serum-liver compartment was on average 130 % of that of the controls. This suggests that the equilibration of newly formed hepatic cholesterol with serum and with extravascular pools was decreased, and that cholesterol synthesis from mevalonate was not decreased to any appreciable extent. However, activation of the feedback mechanism by dietary cholesterol resulted in a marked reduction in the conversion of ^{14}C-acetate to serum cholesterol. The ^{14}C ^{3}H ratio in the lower part of Fig. 1 and in Table 3 decreased 5- and 2-fold respectively. Assuming that the dilution of ^{14}C-cholesterol was the same as that of ^{3}H-cholesterol, the change of the ratio indicates that cholesterol feeding reduced serum cholesterol synthesis between acetate and mevalonate by 80 % and 50 % respectively.

The fraction of the radioactivity from ^{3}H-leucine found in the serum and liver cholesterol 20 hr after injection of the label was about a quarter of that from ^{14}C-acetate and only about 0.3 % of that from ^{3}H-mevalonate (Table 3). Thus

TABLE 3

Recovery of labels (% of dose) in cholesterol of serum and liver pools 20 hr after administration of ^{3}H-mevalonate ^{14}C-acetate and ^{3}H-leucine ^{14}C-acetate mixtures intraperitoneally to control and cholesterol-fed rats.

Tissue	Mevalonate/acetate experiment				Leucine/acetate experiment			
	Total cholesterol	^{3}H-cholesterol	^{14}C-cholesterol	^{14}C ^{3}H	Total cholesterol	^{3}H-cholesterol	^{14}C-cholesterol	^{14}C ^{3}H
	mg	% of dose	% of dose	x10^{-3}	mg	% of dose	% of dose	
				Control rats				
Serum *	4.2	0.951	0.0123	12.9	4.9	0.0028	0.0165	5.9
Liver	29.6	6.000	0.0564	9.4	29.3	0.0144	0.0666	4.6
Total	33.8	6.951	0.0687	9.9	34.2	0.0172	0.0831	4.8
				Cholesterol-fed rats				
Serum *	5.9	0.878	0.0060	6.8	7.3	0.0032	0.0132	4.1
Liver	42.6	8.155	0.0366	4.5	47.6	0.0166	0.0600	3.6
Total	48.5	9.033	0.0426	4.7	54.9	0.0198	0.0732	3.7

* Serum volume assumed to be 4 % of body weight.

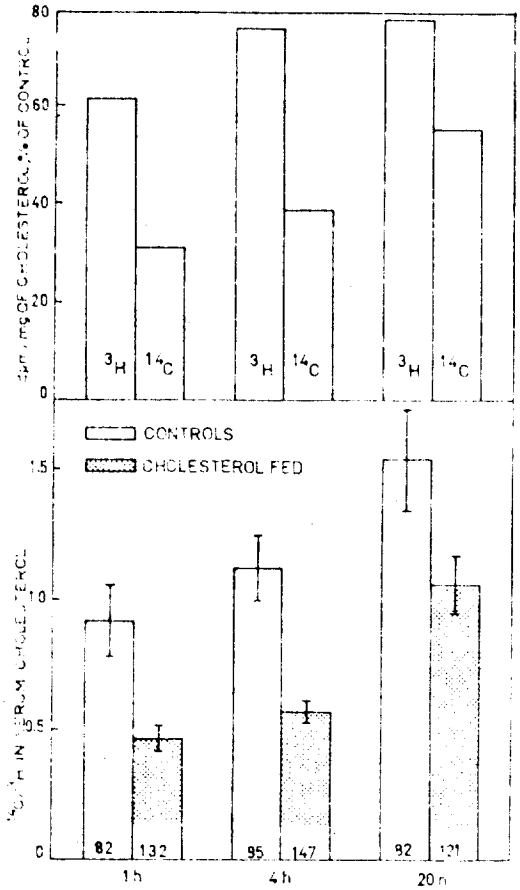

Fig. 2. Effect of dietary cholesterol on conversion of a ^{14}C-acetate-^3H-leucine mixture to serum cholesterol in rats on a fat-free diet. Upper panel shows specific activity of serum cholesterol in treated rats in per cent of that in rats on the fat-free diet. ^{14}C ^3H ratio in total serum cholesterol in the lower panel. Figures at the base of each bar indicate serum cholesterol concentration (mg 100 ml). Each group consisted of seven animals. Same rats as in leucine-acetate experiment of Table 3.

indicates, however, that leucine is a significant precursor of serum and liver cholesterol, particularly because ^3H-leucine may have been considerably more diluted by its non-labelled endogenous pool than ^{14}C-acetate and ^3H-mevalonate by their respective pools.

The specific activity of serum ^3H-cholesterol formed from ^3H-leucine was reduced significantly when a mixture of ^3H-leucine and ^{14}C-acetate was administered to cholesterol-fed animals (Fig. 2). However, the total amount of ^3H-cholesterol in the serum-liver compartment at 20 hr was 114 % of that in controls. This suggests, as in the case of mevalonate, that the reduced specific activity was mainly caused by expanded pool size, and perhaps to a lesser extent by inhibited synthesis. The specific activity of ^{14}C-cholesterol was reduced more markedly, so that the ^{14}C ^3H ratio of serum cholesterol (lower part of Fig. 2) was lower in cholesterol-fed than in control animals. But the decrement of the ratio in both liver and serum cholesterol 20 hr after injection of the labels is less than that found after injection of mevalonate plus acetate. Acetate incorporation was presumably similarly inhibited in both groups of rats. Therefore the different change of the ratios in the liver cholesterol suggests that the hepatic conversion of leucine to cholesterol was slightly inhibited by cholesterol feeding, this inhibition being less than that of acetate. Although this interpretation agrees with the in vitro results (Table 4) it is possible that the relative inhibition from leucine and acetate was the same in the liver in vivo, the portion of the serum and liver cholesterol formed from leucine by extrahepatic, feedback-insensitive tissues, being higher than that formed from acetate. The change of the ratio in the leucine-acetate experiments indicates, in any case, that feedback-insensitive tissue(s) produce relatively more cholesterol from leucine than from acetate. An increase of the ratio after 1 hr, particularly in cholesterol-fed rats, suggests that release of newly formed ^3H-cholesterol into the serum is faster than that of ^{14}C-cholesterol.

DISCUSSION

The liver is known to have a greater potential capacity for synthesizing cholesterol from acetate in vitro than any other organ of the rat (3). The present in vitro experiments suggest that under the conditions employed the ability of the total muscle tissue to synthesize cholesterol from leucine was more than four times that of any other organ to synthesize it from mevalonate, acetate and

TABLE 4

*Abilities of different organs to synthesize cholesterol from various precursors in vitro (dpm x 10³/whole organ/2 hr)**

Organ	Total weight	Leucine	Mevalonate	Acetate
Muscle	30	238	10	1
Liver	6	15	59	36
Intestine	3	14	14	1
Kidney	1.5	14	5	0.2
Total		281	88	38.2

* Calculated from data of Table 1 without taking into consideration that three moles of acetate are needed to form one mole of hydroxymethyl glutarate (synthesized from one mole of leucine) or mevalonate.

leucine (Table 4). In vivo, however, the contribution of leucine to serum cholesterol was less significant. This suggests that there was a marked dilution of the label, and that the bulk of the cholesterol synthesized by extrahepatic tissues remained bound to cellular structures, less being released into the circulation. The concentration of serum leucine is about 13 μmoles 100 ml (25), while the intracellular level may be as much as 30—50 times higher (3). The serum acetate level is only about 4 μmoles 100 ml (4), the mevalonate pool apparently being zero (8). That circulating leucine is rapidly catabolized via the HMG pool is indicated by a finding that within 30 min. 40 % of the ^{14}C administered intraperitoneally as carboxyl-labelled leucine is expired as $^{14}CO_2$ (22).

A low hepatic transaminase activity (10), limiting the conversion of leucine to α-ketoisocaproate and further to HMG, might explain the low conversion of leucine to cholesterol in the liver in vitro as compared to that in muscle tissue. This enzyme is mainly in the membranous fraction of cells (2, 21). Centrifugation of the homogenate in the experiments in Table 2 may have reduced its activity in the supernatant, thus explaining the low cholesterol production from leucine as compared with that in Table 1. The consumption of acetate primarily for oxidation, and poor uptake of mevalonate by subcellular fractions in extrahepatic tissues, probably explain the low conversion of these precursors to cholesterol, particularly because utilization of leucine clearly demonstrates that these organs are able to produce cholesterol at a relatively high rate, provided that a suitable precursor is available.

Cholesterol feeding is known to inhibit its own endogenous synthesis in the liver (5, 7, 28) by activating the negative feedback mechanism between HMG and mevalonate (26, 27), i.e. the activity of microsomal HMG-reductase is inhibited by dietary cholesterol (13). Thus the incorporations of acetate and leucine into cholesterol should have been reduced equally, because they have a common intermediate, HMG. The incorporation of ^{3}H-leucine was actually inhibited less than that of ^{14}C-acetate in the liver slices and homogenate. This may have been due to conversion of a relatively high proportion of ^{3}H-HMG to mevalonate via a pathway which is not under feedback control, while most of the ^{14}C-HMG was metabolized through the feedback sensitive route. It is known in fact that the synthesis of mevalonate takes place in at least two subcellular compartments, less actively in the soluble fraction, which is not under feedback control, and at a higher rate in the microsomal fraction, under a sensitive feedback mechanism (27). According to the in vitro studies (Table 4) 40—50 % of the leucine which is converted to cholesterol is metabolized through the former route, and 50—60 % through the latter route, either via the acetate pool or via direct entry of HMG to the feedback-sensitive pathway (Fig. 3).

In the rat, serum cholesterol mainly originates from the liver (20). Thus the

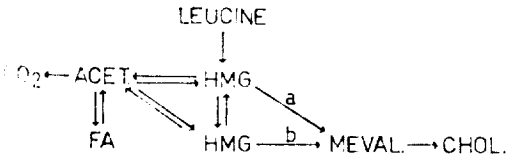

Fig 3. Tentative hepatic metabolism of leucine to cholesterol via feedback-insensitive (a) and -sensitive (b) pathways in the rat. FA = fatty acids, HMG = hydroxymethyl glutarate-CoA.

effectiveness of the feedback mechanism could be measured in vivo by determining the incorporation into serum cholesterol of two simultaneously administered precursors, one before the feedback mechanism (^{14}C-acetate), the other after it (^{3}H-mevalonate) during low and high cholesterol intake (15). Since the basic diet is the same, the acetate pool presumably remains unaltered. Cholesterol-induced reduction in the hepatic conversion of ^{14}C-acetate to ^{14}C-mevalonate will decrease the ^{14}C ^{3}H ratio of serum cholesterol. The changes in synthesis or pool sizes of precursors after mevalonate, probably caused by dietary cholesterol, will similarly affect the incorporation of both labels into cholesterol. Thus, 1) reduced pool size of numerous intermediates between mevalonate and cholesterol, 2) inhibited incorporation of mevalonate to cholesterol and 3) expansion of the exchangeable cholesterol pool will have the same effect on the specific activity of serum cholesterol synthesized either from ^{3}H-mevalonate (administered) or ^{14}C-mevalonate (formed from ^{14}C-acetate). Although no information is available on the possible alterations in pool size of intermediates between mevalonate and squalene, cholesterol feeding decreased the concentration of liver methyl sterols (16). Accordingly, radioactivity originating from ^{3}H- and ^{14}C-mevalonate is diluted less markedly by this sterol group in cholesterol-fed animals, and the specific activity in serum cholesterol may have been increased. However, the possible opposite action of dietary cholesterol in inhibiting synthesis between mevalonate and squalene (6), and of increased dilution on newly formed cholesterol in the expanded cholesterol pool might finally overcompensate for this effect. Therefore, it is apparent that the specific activity of serum cholesterol measured with one isotope could give erroneous information about endogenous cholesterol synthesis. The effects of changes in pool sizes should be corrected for with another isotope.

In the mevalonate-acetate experiments of the present and earlier studies (15) the decrease in the ^{14}C ^{3}H ratio by a factor of 2—5 in cholesterol-fed rats indicates that the synthesis of endogenous cholesterol was decreased by 50—80 %. Thus, in the rat, 20—50 % of serum cholesterol originates either from the feedback-insensitive pathway of the liver or from extrahepatic tissues. These figures are somewhat higher than those obtained by the radioactive cholesterol feeding technique (20), partly because in the latter method the endogenous non-labelled cholesterol is diluted by the expanded pool of exchangeable cholesterol.

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THE EFFECT OF SODIUM ACETATE FEEDING ON MILK AND FAT YIELD, BLOOD SUGAR, AND BLOOD KETONES OF DAIRY COWS[1,2,3]

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It has been shown that a decrease in the level of acetic acid in the rumen contents is associated with a marked depression of milk fat in cows fed a high-concentrate diet with either very limited amounts of long hay or with very liberal amounts of alfalfa meal as the sole roughage *(8, 9)*. In either case, the feeding of sodium acetate caused a recovery toward normal milk fat levels, indicating that the acetates which are normally a product of rumen fermentation have an essential role in the synthesis of milk fat.

A study of the value of sodium acetate feeding for treatment of ketosis has been reported *(5)*. In most cows which recovered after the feeding of sodium acetate, blood sugar increased and blood ketones decreased. However, since under the conditions of the experiment it was not possible to set up an untreated control group, recovery may have been spontaneous rather than specifically due to acetate feeding. Recovery from ketosis is ordinarily accompanied by a rise in blood sugar and a decrease in blood ketones, and the changes in these blood values may have been incidental to the recovery rather than a specific effect of the sodium acetate feeding. Schultz and Smith *(7)* observed a slight increase in blood sugar and no appreciable change in blood ketones after the feeding of acetic acid to goats.

The stimulating effect of acetate feeding to cows with diet-depressed milk fat and rumen acetate raises a question as to the effect upon cows with normal milk fat and rumen acetate levels. Should a comparable increase in milk fat percentage result, it would have practical implications in the production-testing of dairy cows if sodium acetate were fed deliberately as a milk fat stimulant, or if indicated in the treatment of ketosis.

This experiment was designed to determine the effect of sodium acetate, when fed to cows in normal health and on a normal roughage diet, on milk and fat production, milk fat percentage, blood glucose and blood ketones.

EXPERIMENTAL PROCEDURE

A double reversal design was chosen by which it would be possible to detect small differences in blood and milk values resulting from the feeding of sodium

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acetate *(3)*. In each of the three 10-day periods, the first 4 days were used as a transition period and the last 6 days for the comparison. Eighteen cows of four breeds were paired according to breed, stage of lactation, location in barn, age, milk production, and fat test. Cows of each pair were then assigned at random to Group A or B.

TABLE 1

The effect of feeding 1 lb. of sodium acetate daily on milk and fat production, milk fat test, blood glucose, and blood ketones of 18 cows

	Average fat test	Average fat/day	Average milk/day	Blood glucose	Blood ketones
	(%)	*(lb.)*	*(lb.)*	*(mg. %)*	*(mg. %)*
Control	4.743	1.346	28.52	58.056	2.772
Sodium acetate	4.822	1.360	28.39	58.092	2.742
Difference	0.079	0.014	−0.13	0.036	−0.030
LSD (0.05)[a]	0.102	0.065	0.84	0.609	0.183

[a] LSD (0.05) = least significant difference at 0.05 level.

The concentrate mixture, fed at the level of 16 lb. per day to all cows, consisted of 700 lb. each of ground oats and corn, 400 lb. of wheat bran, 200 lb. of linseed or soybean oil meal, 20 lb. of steamed bone meal, 20 lb. of trace mineralized salt, and ½ lb. of irradiated yeast. During the period of sodium acetate feeding, each cow received 1 lb. of technical grade sodium acetate daily, incorporated in the concentrate mixture. In a number of instances individual cows at one or more feedings refused to eat the grain containing sodium acetate. When this occurred the sodium acetate was given separately in gelatin capsules. The cows were given a liberal feeding of corn silage in the evenings and allowed free access to alfalfa-grass hay during the day.

Butterfat tests were made of each individual milking by the Babcock method. Blood samples were taken from the jugular vein on the first and fifth days of each 10-day period. Potassium oxalate and sodium fluoride were used as anticoagulant and preservative, respectively *(3)*. Protein-free filtrates of the whole blood were prepared by the Folin-Wu method *(4)*. Blood ketones and glucose were determined by methods outlined by Behre *et al. (1)* and Nelson *(6)*, respectively.

Total milk and fat production for the 6-day periods was determined for each cow by adding the daily milk and fat weights. The average fat test was then calculated. The values for blood glucose and ketones were determined by averaging the values obtained for the samples taken on the fifth and tenth days.

RESULTS AND DISCUSSION

There were no significant differences in milk fat test, total milk or fat production, blood glucose, or blood ketones between the cows receiving the control ration and those receiving 1 lb. of sodium acetate daily (Table 1). The sensitivity of the experiment, as indicated by the least significant differences, was sufficient to detect any differences of importance.

It seems that there is a minimum level of acetate required for production of milk of normal fat content and that feeding of acetate up to this amount will cause recovery from the diet-depressed milk fat, but additional amounts above this minimum level will not cause further increases. The feeding of sodium acetate to cows on production test should not contribute to inaccuracy of the records.

SUMMARY

The effects of feeding 1 lb. of sodium acetate daily for 10-day periods on milk and fat production, milk fat test, blood glucose, and blood ketones of normal cows receiving normal diets were determined. There were no significant differences ($P = 0.05$), in any of the values studied, between the cows receiving acetate and the controls. The sensitivity of the experiment was sufficient to detect average differences of 0.1% in fat test, 0.06 lb. of fat, or 0.8 lb. of milk per day, 0.6 mg. % blood glucose or 0.18 mg. % blood ketones.

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The Treatment of Ketosis in Dairy Cows by Oral Administration of Sodium Acetate

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IN A STUDY, with dairy cows, of the relationship of the amount and physical state of the roughage fed to the fat content of their milk, Tyznik[6] found that the acetates produced by the rumen microörganisms seem to play an essential role in the synthesis of milk fat. On an extremely low roughage and high concentrate ration, the fat content of the milk and the acetic acid content of the rumen ingesta were depressed simultaneously, with an immediate recovery of the milk fat level when sodium acetate was fed. The quick response of the milk fat test to feeding of acetates suggested that they might be of therapeutic value in cases of ketosis.

Some of the lower fatty acids, including acetic acid, are absorbed directly from the rumen into the blood stream, thus becoming available more quickly than most other materials given orally. Acetic acid is a readily metabolized source of energy which is described by Hawk, Oser, and Summerson[1] as occupying "a key position in the metabolism of not only fatty acids but also carbohydrates and certain amino acids."

Schultz, Smith, and Lardy[5] found that acetic and propionic acids administered orally or intravenously caused no increase in blood ketone levels of normal goats, but butyric, caproic, caprylic, and capric acids caused marked increases.

Schultz and Smith[4] found that butyric acid, given orally, caused depressed blood sugar, with increased blood ketones. Propionic acid caused a sharp increase and acetic acid a slight increase in blood sugar. Plaut and Smith,[2] using a radioactive caproate, have shown conversion of caproate to acetoacetate. These reports have indicated that the fatty acids produced by the rumen microörganisms may play a part in the occurrence of ketosis in ruminants. Schultz[3] has reported encouraging results from feeding sodium propionate to cows with ketosis.

Tyznik in 1951[6] fed sodium acetate to 6 dairy cows, all of which showed definite clinical signs of ketosis and a positive qualitative urine reaction. Four which had received no previous treatment recovered after feeding ¼ lb. of sodium acetate daily for four successive days. The urine test became negative in two to five days. Another, fresh six weeks, had been treated four times with glucose and once with chloral hydrate, but had relapsed after each treatment. After four daily feedings of ¼ lb. of sodium acetate, she recovered from clinical signs and had a negative urine test. The sixth cow had relapsed after five treatments with intravenous glucose and had severe clinical signs. She was fed ½ lb. of sodium acetate daily for seven successive days. Within eight hours after the first feeding, she was on her feet and eating; and in seven days recovery was complete, including a negative urine test. None of these cows suffered any recurrence. Recovery of appetite and milk production was striking.

A cooperating dairyman who was encountering frequent cases of ketosis treated 5 cows in early lactation with one or more feedings of sodium acetate. All recovered from clinical signs, with a marked stimulation of appetite and milk production and a negative urine reaction in all except 1. Two cows in other herds, both showing clinical signs and positive urine tests, were treated by feeding sodium acetate. One recovered from clinical signs after two days of feeding ¼ lb. of sodium acetate daily. The other, which had metritis, previously had failed to respond either to glucose or to sodium acetate. After being fed 1 lb. of sodium acetate daily for twenty days, she recovered completely from clinical signs and had a negative urine test, but a simultaneous recovery from metritis may have been responsible to a greater degree than the treatment.

PRESENT STUDY

To secure a more accurate evaluation of sodium acetate as an aid to treatment of ketosis, 34 additional cases were studied in eight herds in which blood and urine samples could be taken. Blood sugar and blood and urine ketones were determined on samples taken before acetate was given, and periodically thereafter. A qualitative urine test also was run on most of the urine samples. These cases varied in severity, but only clear-cut cases, as judged by clini-

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cal signs and urine tests, are reported. The results are summarized in table 1.

Of these 34 cases, 16 recovered in less than two weeks and 2 others in less than three weeks with no treatment other than oral sodium acetate. The amounts fed varied from ¼ to 1 lb. daily. In the following 6 cases, the effect of the acetate could not be appraised: in 3, because of other treatment; in 1, because it was not possible to observe the cow after her reported recovery; case 20, because of complication by metritis (while her general condition was improving, the blood picture did not indicate recovery); and case 19, because of a severe gangrenous pneumonia, with an abnormally high blood sugar (blood and urine ketones returned to a normal level following treatment, even though she was not eating, and death ensued after the third day).

In the remaining 10 cases, recovery did not occur within a reasonable time. Nine of these also had glucose therapy, to which only 1 responded; 2 of them also showed no improvement from sodium propionate given orally. Two had uterine infections and did not recover from signs of ketosis until the infections subsided. Five of the 10 recovered when put on good pasture.

In general, the blood sugar increased and blood and urine ketone values decreased with the progress of recovery.

Four cows in another herd, which manifested clinical signs of ketosis, recovered when given ½ lb. of sodium acetate daily for four successive days. Their blood and

TABLE 1—Effect of Sodium Acetate on Blood Values of Cows with Ketosis

Case (No.)	Before treatment: Milk lb.	Blood sugar mg.%	Blood ketones mg.%	Urine ketones mg.%	After treatment: Milk lb.	Blood sugar mg.%	Blood ketones mg.%	Urine ketones mg.%	Sodium acetate: Period fed (days)	Total amount lb.
RECOVERED WITHOUT OTHER TREATMENT										
1	36	41	9	97	35	52	4	12	6	1.75
2	35	34	29	....	40	57	5	7	5	4.50
3	43	53	13	152	57	61	4	4	8	7.50
4	25	36	15	....	32	52	4	22	11	5.50
5	45	34	16	132	42	51	5	21	9	2.50
6	35	46	12	148	38	52	5	38	13	2.50
7	38	42	19	65	54	62	4	11	11	2.63
8	....	35	31	335	....	58	4	6	17	12.00
9	48	47	25	146	....	59	7	....	20	17.00
10	42	39	17	154	58	86	2	7	10	9.50
11	....	60	8	....	....	52	4	....	3	2.00
12	37	59	3	28	41	60	2	5	4	1.00
13	....	45	4	15	....	52	3	8	2	0.50
14	48	58	9	18	72	59	3	10	7	6.00
15	50	44	7	45	53	51	7	24	4	3.50
16	42	50	6	91	49	50	6	11	8	8.00
17	49	42	4	75	83	50	5	20	7	6.00
18	....	....	..	23	..	57	2	5	1	0.50
RESULTS QUESTIONABLE DUE TO COMPLICATIONS, INCOMPLETE INFORMATION, OR OTHER TREATMENTS										
19(a)	....	93	16	85	....	104	6	3	3	1.50
20(b)	70	....	56	....	72	59	30	....	13	7.50
21(c)	35	50	13	196	36	44	13	145	7	3.00
22(d)	66	56	5	....	63	52	3	....	7	6.50
23(d)	51	32	32	....	52	53	36	....	5	5.50
24(d)	35	46	29	821	47	61	7	27	11	9.50
NO APPARENT BENEFIT FROM SODIUM ACETATE										
25(b,e)	64	51	21	52	68	45	23	61	5	4.50
26(f)	47	38	8	73	44	46	12	74	25	10.25
27(b,e)	39	31	11	74	30	41	8	48	7	5.00
28(e)	28	38	14	205	24	35	37	648	37	6.75
29(e)	66	46	27	....	72	51	18	....	16	15.00
30(e)	61	28	20	138	63	43	12	311	22	11.50
31	35	46	21	234	....	40	30	331	14	8.50
32(e)	30	42	19	82	....	56	34	145	7	7.00
33(e,g)	52	40	34	271	58	34	14	540	5	3.50
34(e,g)	56	48	19	213	45	45	32	394	5	3.50

(a) Ketosis secondary to pneumonia with elevated temperature and high blood sugar — died after third day; (b) metritis; (c) no opportunity for later samples — owner reported recovery without further treatment; (d) also received glucose therapy — recovered from clinical signs; (e) also failed to respond to glucose therapy; (f) recovered following two intravenous glucose treatments; (g) also failed to respond to sodium propionate, given orally.

urine were not analyzed and they are not included in table 1.

DISCUSSION

It is difficult to evaluate accurately any treatment for ketosis. Since it is not possible to produce experimentally the condition as it occurs in the field, the investigator is limited to naturally occurring cases, which necessitates coöperation in privately owned herds where part of the animals can not be left without treatment to serve as controls, nor can other treatment be forbidden.

Probably a fair percentage of animals would recover spontaneously within a reasonable time; consequently, the value of any treatment is likely to be overestimated. Satisfactory information on the frequency or pattern of spontaneous recovery is not available to provide a standard for evaluating the effectiveness of treatment. Furthermore, ketosis occurs in varying degrees of severity and may be complicated by various contributing factors.

In the cases reported, there was no selection except that those with a questionable diagnosis were omitted. The blood ketone level of some of the cases shown in table 1 was within a range ordinarily considered normal, but all showed definite clinical signs with the odor of acetone on the breath, anorexia, and positive qualitative urine reaction.

The frequency of recovery was sufficiently high to indicate either that the acetate treatment was beneficial or that there may be reason to question the justification for routine treatment. The pattern of recovery in relation to treatment indicated that the sodium acetate contributed to recovery. Only 1 of the 10 which did not respond to acetate responded to other treatment.

The sodium salt of the acetate was used, since it is inexpensive, readily obtainable, and not unpalatable to most animals. Since sodium compounds from saliva are the principal buffering agents in the rumen, the fatty acids produced by rumen fermentation are probably absorbed as sodium salts. These fatty acid compounds appear to be absorbed directly from the rumen, reaching the blood stream quickly.

The acetate, a readily metabolized source of energy, may have a sparing action on blood sugars, or may contribute directly to complete oxidation of the fats. The sodium, by increasing the alkali reserves of the blood, may contribute to relief of any acidosis caused by the ketones in the blood stream.

Also, sodium acetate seemed to have a stimulating effect on the appetite of some animals. It is nontoxic, as indicated by a trial with 18 normal cows fed 1 lb. per head daily during ten-day periods.

Sodium acetate should be compatible with other treatments and may have value when used in combination with them. While in some cases the apparent response to acetate feeding was almost immediate, usually the reaction was slower than with glucose therapy. The frequency of ketosis recurrence following a single intravenous treatment with glucose suggests that oral sodium acetate might be useful following glucose therapy. Favorable results from such use in a few cases have been reported by a coöperating veterinarian. More observations are needed for adequate evaluation of this treatment.

SUMMARY

Sodium acetate was fed at rates varying from ¼ to 1 lb. daily to 34 cows showing signs of ketosis. Blood sugar and ketones and, in most cases, urine ketones were determined.

Of the 34 cases, 18 recovered with no other treatment, 6 were confused by complicating factors, incomplete information, or by other treatment given simultaneously, and 10 failed to respond. Of the 10, 8 also failed to respond to intravenous glucose treatment, and 2 of the 8 to sodium propionate feeding. Only 1 of the 10 responded to glucose therapy.

Fifteen other cows from which blood samples were not analyzed made satisfactory recoveries from clinical signs following sodium acetate as the sole treatment.

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Acids as Poultry Meat Preservatives

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PRESERVATION of poultry carcasses by the use of edible acids has been demonstrated by several workers. Among the acids which have been investigated are sorbic, hydrochloric, lactic, and citric acids.

Three groups have reported on the use of sorbic acid. Meyer *et al.*, (1959) in work with edible coatings reported that when sorbic acid was combined with agar, gelcarin or lipid coatings, shelf life was increased. Two years later, Kaloyereas *et al.*, (1961) reported that flake ice containing glycol diformate and sorbic acid had some effect on extending the shelf life of poultry meat.

Perry *et al.*, (1964) developed a method in which carcasses were first immersed in a slush ice solution of sodium dihydrogen phosphate for 2 hours to lower the surface pH below 5.5. Then after draining, the parts were sprayed with a 71°C. solution of 7.5% sorbic acid dissolved in a 70% propylene glycol, 20% water, 10% glycerine solution. Using this method, these workers were able to extend the shelf life of poultry carcasses 13 days.

Lactic acid has also been used to lower bacterial counts. Silliker *et al.*, (1960) inoculated chill water with Lactobacillus

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cells at a concentration of 100 × 10^6 cells/ml. By using this treatment, they were able to obtain an additional 3-day extension of shelf life. Murphy and Murphy (1963) observed that chill water containing concentrations of 0.05 to 0.25% lactic, citric, or hydrochloric acids brought a lowering of the bacterial count of the chill water and the surface of the carcasses. They reported that the acid treatment was especially effective against *Pseudomonas* organisms and that Staphylococcus and *coliform* organisms which cause food poisoning were also eliminated.

The effect of hydrochloric acid on the growth of microflora found on poultry carcasses has been reported by Mountney *et al.* (1964). They reported that the shelf life of poultry can be extended by adding 50 p.p.m. of HCl to the chill water.

One problem in the use of acids, especially at low pH values is their effect on flavor. Kazeniac (1961) in work in which lactic acid was added to chicken broth reported that lactic acid, being a natural component of chicken broth, contributes to good flavor and mouth satisfaction.

In view of the past work on the use of acids as preservatives for poultry carcasses and other foods, the present experiment was designed to determine the effect of different acids and different concentrations of acids on prolonging the shelf life of poultry carcasses and to obtain information which might help to explain the characteristics of these acids which are responsible for their inhibitory effects.

MATERIALS AND METHODS

Legs and thighs from warm, subscalded, broiler carcasses obtained from a local processing plant were removed in one piece from the carcass and the skin pulled over the cut surfaces and held in place with toothpicks. These parts were then agitated together in a peptone salt water solution on a mechanical shaker at 20°C. for 15 minutes, drained 30 seconds and then immersed in unbuffered solutions at 2°C. of either acetic, adipic, citric, fumaric, hydrochloric, lactic, malonic, phosphoric, succinic or sorbic acids for two hours. All solutions except sorbic which was pH 3.1 were at pH 2.5. Sorbic acid is not soluble in water at the concentration required for a pH of 2.5. After the carcasses were removed from the solutions pH determinations were made again to determine the influence of the meat on the solutions. The pH levels at this time were acetic, 2.9; adipic, 2.9; citric, 2.6; fumaric, 2.6; hydrochloric, 2.6; lactic, 2.9; malonic, 2.6; phosphoric, 2.8; succinic, 2.7; and sorbic 3.4. Those acids which inhibited the greatest numbers of microorganisms were tested further at pH 3.0, 3.5 and 4.0 respectively. In addition to the acids, a control sample immersed in distilled water and a sample immersed in 10 p.p.m. of Aeronize (chlortetracycline) were used as reference standards. After chilling, the parts were removed from the respective acid solutions, drained for 30 seconds and then stored individually in polyethylene bags at 2°C.

Total plate counts were made at two day intervals by swabbing a 2cm.^{2} area with a cotton swab moistened with peptone salt water and then inserting and breaking the swab in a dilution blank containing 99 ml. of sterile peptone salt water. A different area of the thigh was swabbed each time. After further dilutions and triplicate plates from each dilution were made, the plates were incubated 48 hours at 20°C. and counts made with a Quebec colony counter.

Odor scores were made independently by three members of the laboratory staff at two day intervals prior to making bacterial counts.

Taste tests were conducted by a panel

TABLE 1. *Comparative effectiveness of edible acids in extending shelf life beyond control samples as measured by first indications of objectionable odor and bacterial counts of 10^7*

Compound	Acid pH 2.5	Odor pH 2.5	10^7 Count pH 2.5
	Percent[2]	Days	Days
Acetic	0.012	6.0	6.0
Adipic	1.500	6.0	6.0
Succinic	1.000	6.0	6.0
Citric	0.300	4.0	3.0
CTC (Aronize)		3.0[1]	3.0[1]
Fumaric	0.200	2.5	0.0
Malonic	1.000	2.0	2.0
Sorbic	Not sol.	2.0	0.0
Hydrochloric	0.010	2.0	2.0
Phosphoric	0.015	1.0	0.0
Lactic	0.275	0.0	0.0

[1] pH 3.0.
[2] Percent concentration at pH 2.5.

with previous taste panel experience on chicken broth and samples of meat from chicken thighs treated with distilled water, adipic acid at pH 2.5 or 3.0, or succinic acid at pH 2.5 or 3.0. The pH of the broths were 5.1, 5.8, 5.3, 6.3, and 6.5 respectively. Broth was made from samples cooked with 1½ times the weight of the meat. Members of the panel were asked to arrange the 5 samples in the order of their preference.

RESULTS

Table 1 lists the acids in order of their effectiveness in inhibiting the growth of microflora on poultry carcasses and also some of their characteristics. The number of days shelf life was extended beyond the control sample is shown in Table 1.

Fig. 1 and 2 illustrate the preservative effects of the several acids at pH 2.5. In those cases where no counts are shown on the graph for a particular acid, counts were less then a log value of 2.5. These same tests were conducted using acetic, adipic, hydrochloric, lactic and succinic acids at pH 4.0. At that pH no preservative action was observed. The acids appeared to exert their greatest inhibitory effect by killing large numbers of organisms during the two hour immersion period and by extending the lag period of growth.

Acetic, one of the most effective inhibitors, was found to be unsuitable for use as a preservative for poultry meat at the concentrations required because the skin of the carcass became hard, leathery, and glistening as a result of protein denaturation. Acetic acid has a chain length of two and contains a single carboxyl group. With the exception of HCl it has the lowest molecular weight of all the acids tested.

Adipic and succinic acids are equal to acetic in their ability to inhibit microorganisms. Both acids have two carboxyl groups. Adipic has a chain length of five and succinic, two. Of the three, succinic appears to have the best combination of

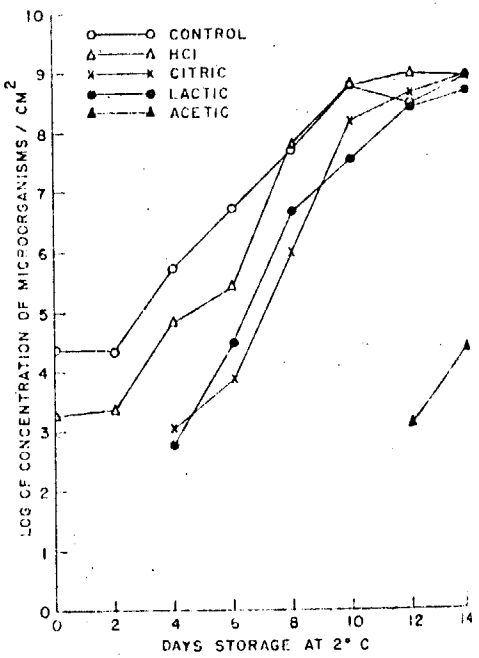

FIG. 1. Effect of selected acids at pH 2.5 on growth of microflora on poultry carcasses.

characteristics for commercial application. Not only is the cost per pound less, but it does not require as much of the dry powder to obtain a pH of 2.5 as adipic.

Acetic, adipic and succinic acids caused the skin of the carcasses to whiten slightly; however, the effect is more pronounced with acetic acid than succinic. The strong odor of acetic acid on carcasses is offensive, while those associated with adipic and succinic acids, although detectable under some conditions, are not unpleasant. In tests where a panel of 7 members were asked to express their preference for broth and meat from unwashed adipic and succinic acid treated carcasses, no significant differences were obtained ($p. < .01$). Washing carcasses in cold running water before cooking removed most of the acid taste.

Hydrochloric, lactic and sorbic acids,

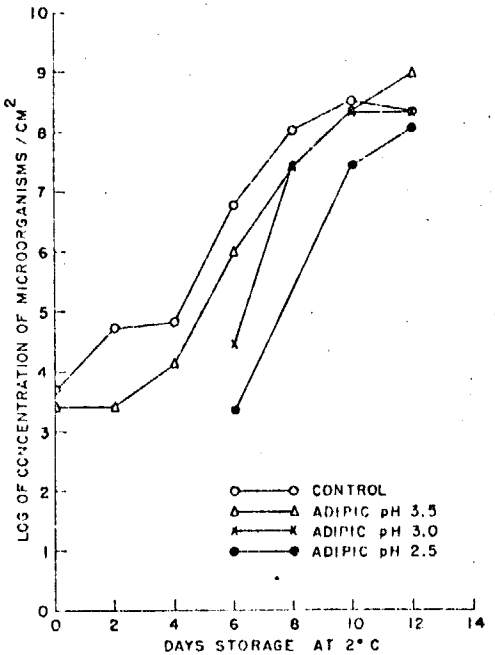

FIG. 3. Effect of pH on growth of microflora, adipic acid.

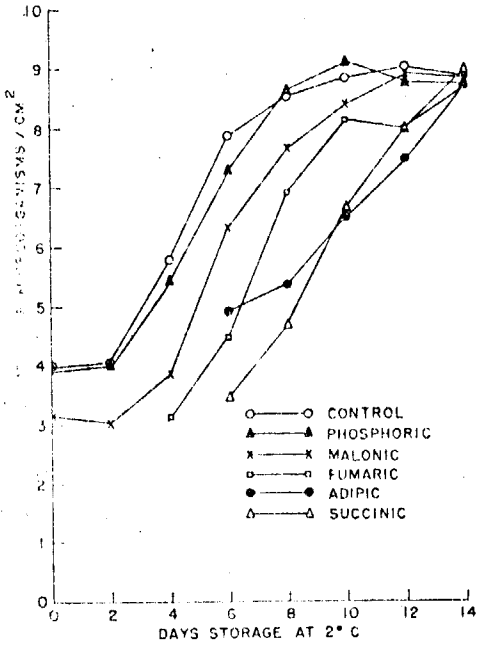

FIG. 2. Effect of selected acids at pH 2.5 on growth of microflora on poultry carcasses.

although they inhibited growth, were not as effective as acetic, adipic or succinic acids in extending shelf life.

Because adipic and succinic acids appear to have promise as preservatives, their effects at different pH values were studied. These are shown in Fig. 3 and 4. Above pH 2.5, inhibition decreased and extension of shelf life declined until at pH 4.0, no inhibition of bacterial growth was observed.

SUMMARY

Ten acids, acetic, adipic, citric, fumaric, hydrochloric, lactic, malonic, phosphoric, and succinic were added to the chill water of cut-up poultry parts in amounts required to bring the pH to 2.5 and one acid, sorbic, to bring the pH to 3.1. Aronize (chlortetracycline) at a level of 10 p.p.m. and distilled water were used as standards for comparison. When measured by

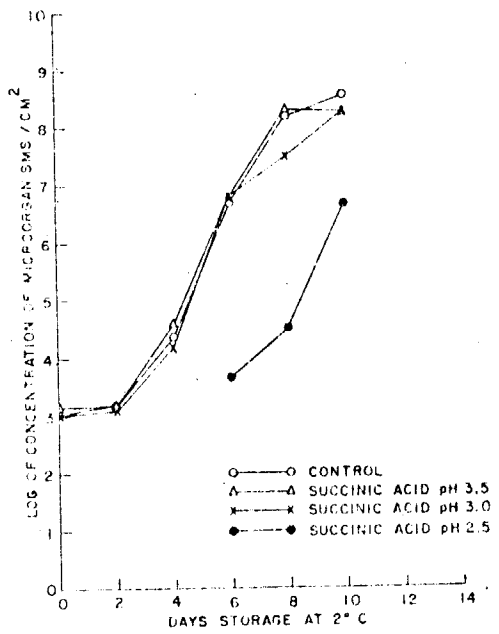

FIG. 4. Effect of pH on growth of microflora, succinic acid.

total numbers of bacteria and number of days to off odor acetic, adipic, succinic, citric, CTC, fumaric, malonic, sorbic, hydrochloric, phosphoric and lactic acids were effective in that order. Acetic, adipic and succinic acids brought about an increase in shelf life of six days more than the control and three days more than CTC. Using adipic and succinic acids, it was observed that as the pH increased from 2.5 to 3.0, 3.5 and 4.0, the inhibitory effects declined until no inhibition was observed at 4.0.

Acetic acid was considered unacceptable because of its pungent odor and its effect on the skin. Adipic and succinic acids gave the best overall results both from their ability to inhibit growth of microflora and still have an acceptable taste. No significant differences ($p<.01$) in preference were found by a taste panel between adipic treated and succinic acid treated samples.

ACKNOWLEDGEMENT

The authors acknowledge with thanks samples of Acronize supplied by American Cyanamid Company and samples of lactic, adipic, fumaric and phosphoric acids supplied by Monsanto Chemical Company.

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Brit. Med. J. 4(5574): 296

Corrosive Poisons

SIR,—I wish to comment on the statement on the treatment of corrosive damage to the stomach made by Drs. S. C. Kennedy and A. E. Bavaki (14 October, p. 93), where the use of magnesium bicarbonate was advocated.

Corrosive poisoning by acetic acid is not a rare occurrence in Ceylon, especially in the rubber-producing areas (acetic acid being used in the coagulation of rubber latex and being available "over the counter"). Our teachers used to impress upon us that magnesium hydroxide or dilute calcium hydroxide should be used locally in the mouth and be given orally whenever the patient could swallow—which was seldom. Bicarbonates were not advocated owing to the theoretically increased risk of perforation due to the evolution of gas, which I believe is a reasonable assumption.—I am, etc.,

London S.W.4. D. R. MUNASINGHE.

Proceedings of the Montana Academy of Sciences 28: 51-57, 1968

EFFECT OF FATTY ACIDS ON *SHIGELLA**

Mitsuru Nakamura and Marilyn Jo Zangar**

The mechanism of pathogenicity in *Shigella* has not yet been clearly elucidated. A number of variables appear to be important; namely, the host, the age of the host, the size of the infective dose, the "strain" and species of the etiologic agent, and the normal intestinal flora. The role of the normal intestinal flora in *Shigella* infections has been reviewed recently (5, 6). There is general agreement that the normal bacterial flora protects the animal against infection and disease due to the *Shigellae*.

Several workers reported that the growth of *Shigella* was inhibited when grown in a mixed culture with other bacteria (1, 4). The associated bacteria apparently produced some chemical or physical change in the environment that affected the growth of the pathogen.

The mechanisms of these interacting reactions become of interest in order to establish the specific role of the normal flora. Hentges reported that *Klebsiella* inhibited *Shigella* in mixed cultures (2). He suggested that the formic and acetic acids produced by *Klebsiella* were responsible for the inhibition of the *Shigella*.

The present investigation was undertaken to study the effects of several fatty acids on the *in vitro* growth of *S. sonnei* and the related species *Escherichia coli* as well as the enteropathogenic variant of *E. coli*. The effects of acetic, propionic, and octanoic acids were studied on *E. coli* B, *E. coli* OB E 0127:B8 (enteropathogenic), *S. sonnei* strain 9453, and *S. sonnei* strain 6761. The bacteria were maintained and exposed to fatty acids in the chemically defined medium (2). The pH of the media containing various concentrations of fatty acids ranging from 0 to 1.8 percent was adjusted to 7.0.

Standard plate counting techniques were used to enumerate the bacterial populations after incubation for 24 hours at 37° C. The activity of the fatty acids on the growth of

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** Undergraduate research participant in microbiology supported by grant GY-2547, Undergraduate Education in Science, National Science Foundation.

E. coli B is illustrated in Figure 1. Octanoic acid was highly toxic and a concentration of 0.3 percent essentially sterilized the culture. Acetic and propionic acids, after producing an initial partial inhibition, did not significantly reduce the growth of this organism even at 1.5 to 1.8 percent. Octanoic acid was the most toxic fatty acid for *E. coli* OB E 0127:B8 (Fig. 2). Acetic and propionic acids were less inhibitory to this strain.

The dose-response curves in Figure 3 and Figure 4 illustrate the activities of the fatty acids against *S. sonnei* strains 9453 and 6761. Octanoic acid was the most bactericidal of the fatty acids. Propionic acid was inhibitory to both strains, although there was a slight difference in the rate of inhibition. The lethal effect of propionic acid was exponential. Acetic acid was highly toxic to strain 6761 but relatively nontoxic to strain 9453 of *S. sonnei*. This is interpreted as due to strain difference in susceptibility to acetic acid.

The three fatty acids studied exerted different inhibitory patterns on the related bacteria studied. According to Hentges the inhibitory activity of acetic acid cannot be attributed to a hydrogen ion effect alone (3). However, the mechanisms by which these fatty acids exert their antibacterial effect are not understood.

Hentges has hypothesized that the fatty acids, particularly acetic acid, are inhibitory for *Shigella* in the intestine (2). It is possible that the fatty acids produced in the intestine by the normal flora inhibit the *Shigella* and thus reduce the possibilities of disease following infection. In view of the fact that our results suggest a considerable variation in susceptibility of *S. sonnei* to acetic acid, we would like to suggest that other mechanisms may operate, besides fatty acid toxicity, in the interaction between *Shigella* and the normal intestinal flora. Furthermore, the acetic acid produced in the intestine will also inhibit *E. coli*, resulting in a much more complex interacting system than merely the inhibition of *Shigella* by the products of another organism.

Currently we are studying the effects of other fatty acids on *Shigella*. We plan to study the effects of fatty acids on *Shigella-Escherichia* hybrids in order to determine if there are phylogenetic relationships among these organisms in relation to fatty acid sensitivity.

Montana Academy of Sciences 28: 51-57, 1968

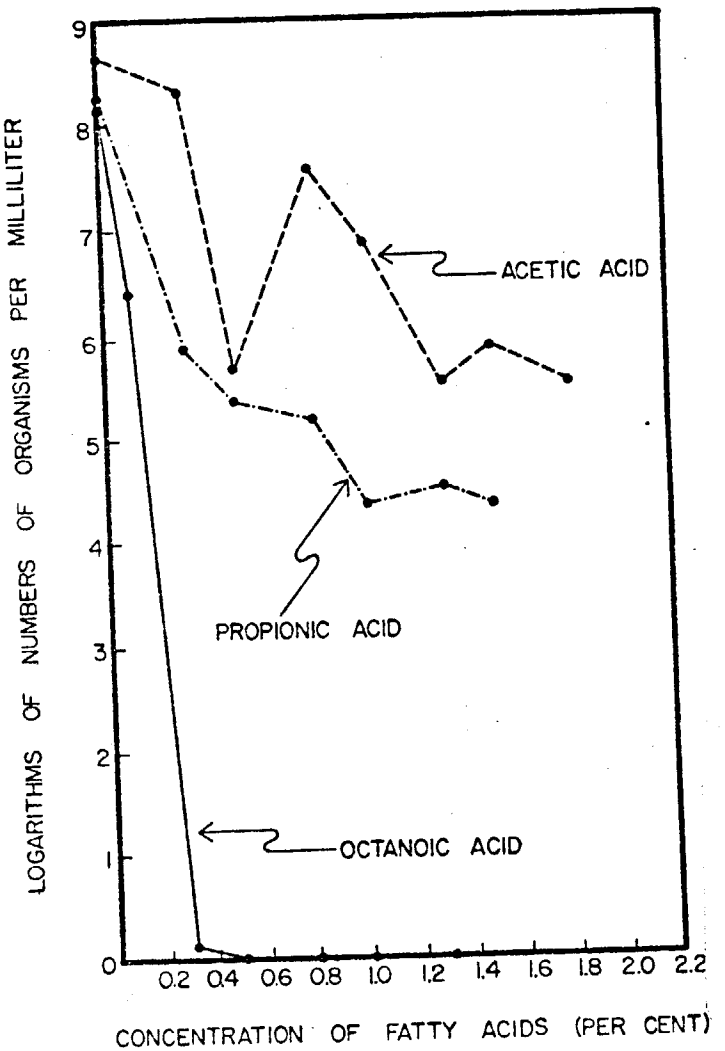

Figure 1. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* B.

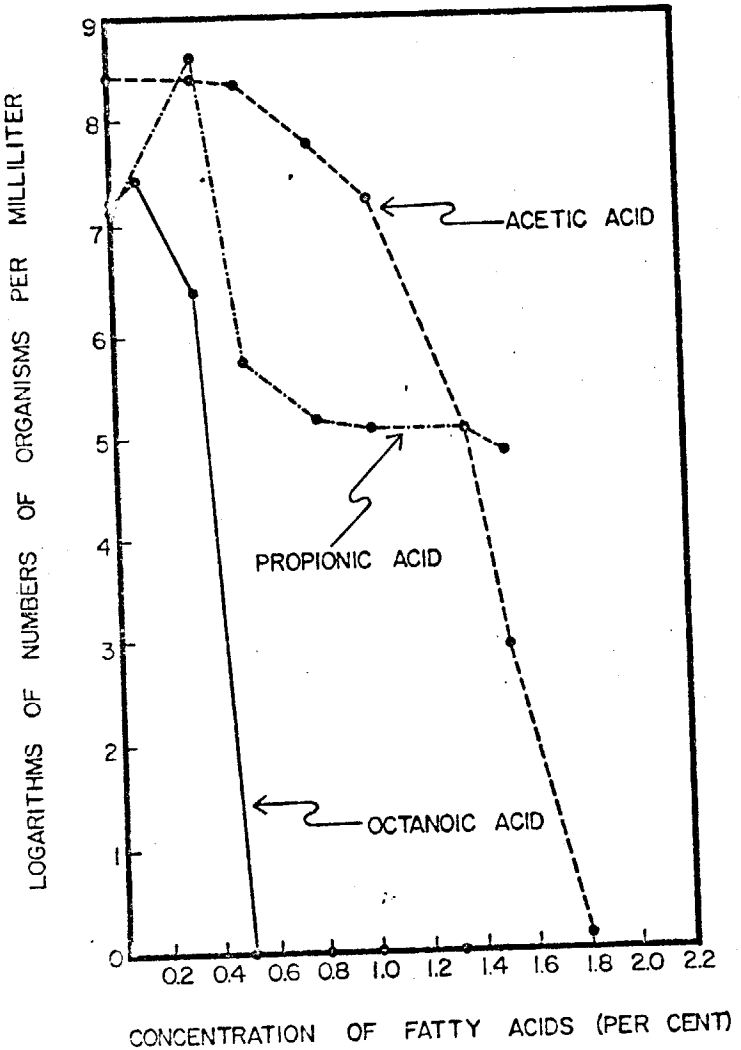

Figure 2. Effect of Acetic, Propionic, and Octanoic Acids on *Escherichia coli* OB E 0127:B8.

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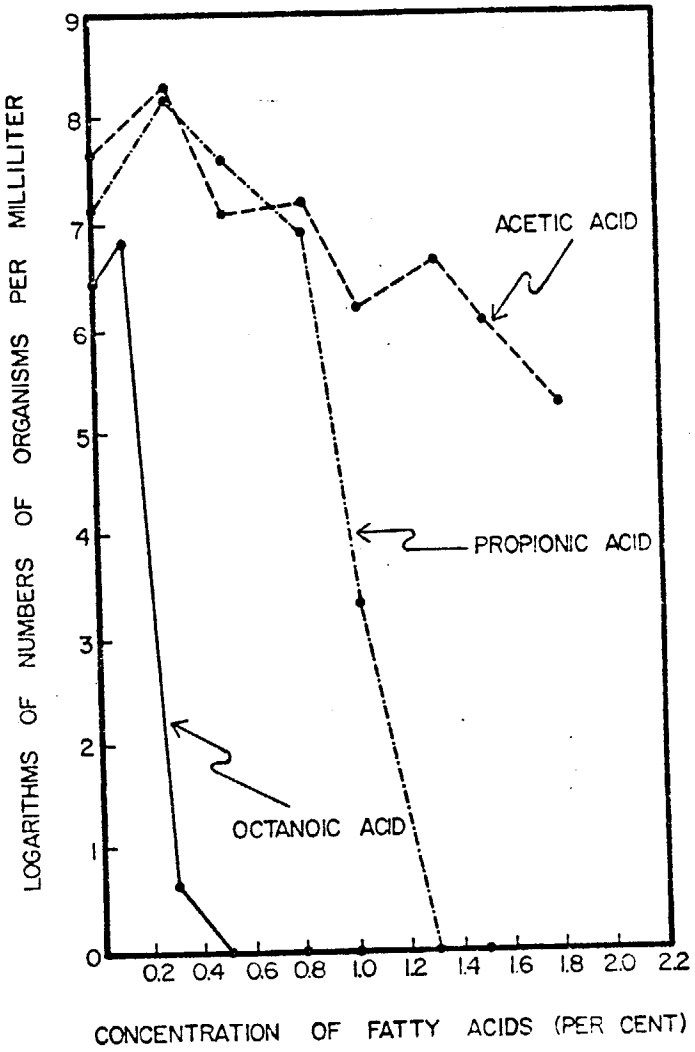

Figure 3. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 9453.

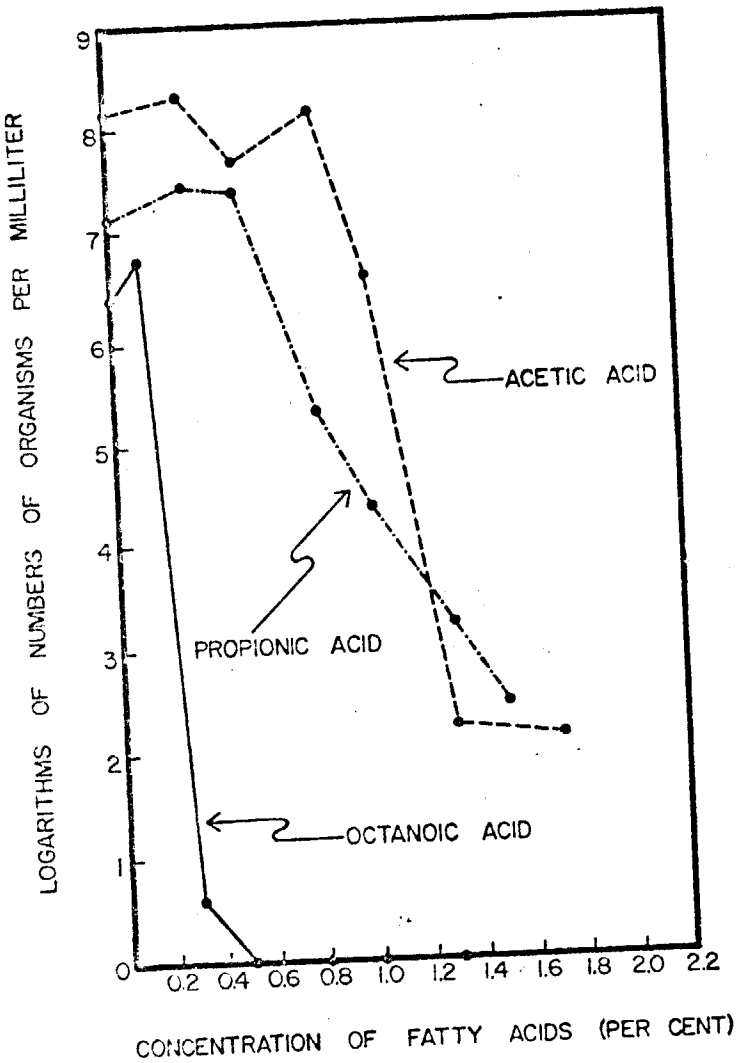

Figure 4. Effect of Acetic, Propionic, and Octanoic Acids on *Shigella sonnei* 6761.

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Department of Microbiology, University of Montana, Missoula

National Academy of Sciences 1972
Annual Poundage Reported Per Substance by NAS and FEMA User Firms
National Academy of Sciences, Wash;ngton, D.C.

Some Organic Acids in Honey[1,2]

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Division of Food Research, Bureau of Chemistry and Soils, Washington, D. C.

The volatile and non-volatile acids in fifteen samples of honey have been determined. The total volatile acids range from 0.011 to 0.051 per cent, and consist mainly of a mixture of formic and acetic acids. Sage honey has the largest amount of acetic acid (0.046 per cent), and tulip honey has the largest amount of formic acid (0.024 per cent). Citric acid accompanied by malic acid was found in all samples. Succinic acid was identified in the samples of higher acidity (sourwood, cotton, and tulip).

THE volatile acids in honey have been mentioned in the literature (*3, 4, 6, 8*), but little has been published as to the nature and amount of the non-volatile acids. Heiduschka (*7*) reports the presence of lactic, malic, tartaric, oxalic, and succinic acids in honey but the methods he used, since they depended on separations and oxidation reactions that were not specific, were not above criticism.

It is well known to honey producers that different floral varieties of honey have marked differences in quality and flavor. In order to ascertain the possible relationship of the organic acids to these flavor differences, as well as to furnish further information on the organic acids present in American honeys, fifteen samples of honey of different floral varieties, produced in 1929, were examined. These honeys were collected from various parts of the United States, with one sample from Hawaii,[3] and were representative of the chief commercial varieties on the American market. There was no evidence of fermentation in any of these samples.

Determination of Non-Volatile Acids

It was the intention to apply the ester distillation method to the separation and identification of the non-volatile acids in honey but, owing to the very small amount of acids present and the large quantity of honey required to yield a sufficient amount of the mixed esters for a fractional distillation, this was not found to be feasible except in the case of tulip honey.

For the separation of the non-volatile acids from each other, an unpublished method was used which was devised by Hartmann and Hillig, of the Food, Drug, and Insecticide Administration. However, this method does not distinguish between malic and succinic acids, so that the results given for malic acid include any succinic acid that may be present.

Citric acid was weighed as pentabromoacetone (*5*), checked for identity by its melting point.

Malic acid was precipitated as the calcium salt, which was ignited and the residual calcium oxide titrated with 0.1 *N* hydrochloric acid. In a few cases, where the amount of material was sufficient, the malic acid determination was checked by the tentative method of the A. O. A. C (*1*).

The non-volatile acids were precipitated from the diluted honeys by lead subacetate. To 5 kg. of honey, diluted with 3 liters of water and 6 liters of alcohol, sufficient lead acetate was added to insure complete precipitation. The precipitate was allowed to settle overnight, the supernatant liquor was siphoned off, and the precipitate was washed with 50 per cent alcohol.

The completeness of the precipitation of the acids as lead salts from the very dilute alcoholic sugar solutions was tested by running a check analysis with known amounts of malic and citric acids corresponding to the average total acidity of the honeys. An 80 per cent solution was prepared from 4 kg. of sucrose, and 5 grams each of malic and citric acid were added. The solution was diluted with water and alcohol and precipitated with lead subacetate exactly as in the case of the honey samples. The total acids recovered from the lead precipitate had an acidity equivalent to 82 per cent of the original acidity. An aliquot of 100 grams of the solution, representing one-fiftieth of the total quantity and containing 0.1 gram each of citric and malic acid, was analyzed. The amount of malic acid recovered was 0.079 gram and of citric acid, 0.079 gram. Therefore, it is safe to assume that the figures for citric and malic acids represent not more than 75 per cent of the amounts actually present.

There was a large titratable acidity in all samples which was not accounted for by the sum of the acids determined. This discrepancy is partly due to incomplete separation of the lead salts from such a dilute solution of the organic acids, but other acids of unstable character, related to the sugars, may also be present, which were not identified.

Determination of Volatile Acids

The volatile acids were determined by steam-distilling 100 grams of honey diluted to a volume of 150 cc., keeping this volume constant, and collecting 1 liter of distillate. The combined volatile acids were obtained by adding an amount of sulfuric acid equivalent to the alkalinity of the ash and distilling a second liter of distillate.

The distillates were titrated with 0.1 *N* sodium hydroxide and evaporated to small volume. The organic acids were liberated with an exact equivalent of sulfuric acid, and the solutions were boiled with mercuric oxide for 20 minutes under a reflux condenser in order to destroy the formic acid.

The solutions, after cooling, were filtered from excess mercuric oxide, acidified with sulfuric acid, and the acids other than formic were distilled and titrated.

The acids sometimes had a faint odor suggesting the presence of higher volatile acids, but in every case, when esterified with ethyl alcohol, the ethyl acetate odor was recognizable.

The total quantities of formic and acetic acid found were corrected for the amounts remaining undistilled according to Dyer (*2*).

Separation of Succinic Acid

Succinic acid was separated from some of the honeys by extracting the honey (diluted with water and acidified with hydrochloric acid) with ether in a continuous-extraction outfit. The partly crystalline residue from the ether was purified by crystallization from water and identified as succinic acid by optical crystallographic data[4] and by the melting point. Tests for tartaric acid gave negative results in all cases.

[1] Received December 12, 1930. Presented before the Division of Agricultural and Food Chemistry at the 80th Meeting of the American Chemical Society, Cincinnati, Ohio, September 8 to 12, 1930.

[2] Food Research Division Contribution 85.

[3] The selection of samples was made with the assistance and advice of the Bee Culture Laboratory of the Bureau of Entomology.

[4] Obtained by G. L. Keenan, of the Food, Drug, and Insecticide Administration.

Results

Malic and citric acids were found in all samples examined. Formic acid, formerly assumed to be an important acid in honey, is present in a relatively small amount, confirming the observations of Fincke (*4*), Heiduschka (*6*), Farnsteiner (*3*), and others.

The results obtained are given in Tables I and II.

Examination of Tulip Honey

In the examination of tulip honey, 4 kg. were diluted with water and alcohol and precipitated with 100 grams of lead subacetate. The precipitate was filtered and washed. Carbon dioxide was passed through a water suspension of the precipitate, and it was then filtered and washed again. The acids recovered from this precipitate were extracted with ether, yielding a crystalline acid which, after recrystallization, was identified by the melting point and optical crystallographic data as succinic acid. The residual acids were converted into ethyl esters, yielding 6 grams of the mixed esters.

This mixture, fractionated at 10 mm., afforded 0.2 gram boiling at 80–125° C., 0.33 gram boiling at 125–140° C., 0.7 gram boiling at 140–160° C., 0.52 gram boiling at 160–170° C., and 2.4 grams boiling at 170° C. Levulinic acid was identified in the lowest fraction by means of its hydrazide. This undoubtedly resulted from the action of the alcoholic hydrochloric acid during esterification on sugars occluded by the lead precipitate. Malic acid was found in the second, third, and fourth fractions by means of its hydrazide, melting at 178–179° C., while the fifth and largest fraction yielded citric hydrazide, melting at 103–105° C. and further identified by optical crystallographic data.

A separate extraction with ether of 200 grams of the honey, acidified with hydrochloric acid, yielded 0.024 gram of purified succinic acid, equivalent to 0.012 per cent.

The predominating non-volatile acid of tulip honey, therefore, is citric acid (about 0.04 per cent). It also has about 0.012 per cent of succinic acid, and a smaller amount of malic acid.

Table I—Volatile Acids of Honey

Variety	Source	Formic Free	Formic Combined	Acetic Free	Acetic Combined	Formic Total	Acetic Total
		%	%	%	%	%	%
Tulip	Md.	0.007	0.017	0.009	0.007	0.024	0.016
Mesquite	Calif.	0.003	0.004	0.002	0.003	0.007	0.005
Sage	Calif.	0.004	0.001	0.039	0.007	0.005	0.046
Orange	Calif.	0.004	0.002	0.003	0.002	0.006	0.005
Sourwood	Va.	0.007	0.004	0.002	0.008	0.011	0.010
Spanish needle	Ill.	0.010	0.012	0.005	0.004	0.022	0.009
Alfalfa	Utah	Nil	0.001	0.007	0.005	0.001	0.012
Clover	Ohio	0.004	0.002	0.002	0.004	0.006	0.006
Star thistle	Calif.	0.004	0.003	0.003	0.002	0.007	0.005
Cotton	Tex.	0.006	0.005	0.006	0.006	0.010	0.012
Sweet clover	Colo.	0.002	0.006	0.003	0.003	0.008	0.006
Fireweed	Wash.	0.006	0.003	0.007	0.006	0.009	0.013
Alfalfa	Calif.	0.007	0.003	0.004	0.003	0.010	0.007
White clover[a]	Ohio	0.007	0.005	0.002	0.005	0.012	0.007
Algarroba	Hawaii	0.002	0.009	0.004	0.010	0.011	0.014

[a] This honey has a green color. The producer states that sweet clover honey or other honey may be mixed with it.

Table II—Non-Volatile Acids of Honey

Variety	Source	Ash	Free Acidity	Alkalinity of Ash	Malic Acid	Citric Acid	Succinic Acid
		%	*Cc./100 grams*[a]		%	%	
Sourwood	Va.	0.28	23.0	30.0	0.003	0.006	Present
Spanish needle	Ill.	0.18	17.3	22.0	Trace	0.001	
Alfalfa	Wash.	0.06	13.5	6.3	Trace	0.001	Trace (?)
Clover	Ohio	0.05	13.0	5.3	Trace	0.001	
Star thistle	Calif.	0.09	23.0	7.5	Trace	Trace	
Cotton	Tex.	0.24	22.5	42.0	0.023	0.008	Present
Sweet clover	Colo.	0.04	10.0	4.3	Trace	0.001	
Fireweed	Wash.	0.07	9.5	8.0	Trace	0.001	
Alfalfa	Calif.	0.17	27.5	20.5	0.002	0.002	
White clover	Ohio	0.11	19.5	13.5	Trace	0.001	
Algarroba	Hawaii	0.52	13.5	37.0	Trace	0.001	
Mesquite	Calif.	0.15	14.0	20.0	0.011	0.007	
Orange	Calif.	0.05	14.9	7.5	Trace	0.001	
Sage	Calif.	0.06	11.5	6.5	Trace	0.001	

[a] Expressed in terms of 0.10 *N* solutions.

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(4) Fincke, *Ibid.*, **23**, 255 (1912).
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(6) Heiduschka, *Z. Nahr. Genussm.*, **21**, 375 (1911).
(7) Heiduschka, *Pharm. Zentralhalle*, **52**, 1051 (1911); *Schweiz. Wochschr.*, **49**, 725 (1911).
(8) Utz, *Pharm. Post*, **41** (6–7), 69 (1908).

EFFECT OF THE PROPIONATE CONCENTRATION ON THE SPECIFIC GROWTH RATE OF *Propionibacterium shermanii*

N. M. Neronova, S. I. Ibragimova, and N. D. Ierusalimskii*

UDC 5.76.8.095.42

Working with a continuous culture of Propionibacterium shermanii, we observed that there is an inverse relationship between the concentration of bacterial biomass and the growth rate (Neronova and Ierusalimskii, 1960). Evidently, this is explained by the fact that an increase in the concentration of biomass is accompanied by a deterioration of the composition of the medium as a result of the accumulation of metabolic products in it and the loss of nutrients, especially lactate. The influence of the residual lactate concentration upon the rate of growth of P. shermanii was investigated in another work (Neronova et al., 1966). This article is devoted to the question of the influence of propionic and acetic acid metabolites upon the growth of P. shermanii.

PROCEDURE

The strain of P. shermanii used in the work was cultured for about a year in a flowing medium with lactate. The medium possessed the following composition: K_2HPO_4 — 1 g, $MgSO_4 \cdot 7H_2O$ — 0.2 g, $(NH_4)_2SO_4$ — 3 g, $CoCl_2$ — 30 mg, 40% yeast autolyzate 80 ml, lactic acid from 1.7 to 2.6%, tap water 1 liter. The pH of the medium was adjusted to 6.7–6.9 with sodium hydroxide. The pH is unchanged during fermentation on this medium, since either one molecule of acetic acid or one molecule of propionic acid (more accurately, their salts) is formed from each molecule of lactic acid. Lactic acid was determined according to Friedeman. Volatile acids were determined by the method of Frolov-Bagreev and Agabal'yants (1951). Propionic acid was determined by Nair's method (Nair, 1932). In the determination both of the total volatile acids and of propionic acid, suitable corrections were introduced for the amount of lactic acid that was distilled off with steam. Acetic acid was determined according to the difference between the total amount of volatile acids and the propionic acid content. To determine the relationship between the concentration of products of fermentation and bacterial growth rate, we used chiefly the method of continuous cultures. At a concentrated flow in the culture vessel (cultivator), a labile equilibrium is established, at which the rate of increase in the microbial biomass is equal to the rate of its removal by the flow of the medium, while the concentration of fermentation products remains at a constant level. This permits a more accurate determination of the relationship of interest to us. In the first series of experiments, the culture was grown for a long time in one cultivator with a 50 ml capacity at different rates of flow. The culture fluid was mixed with nitrogen (Fig. 1). During the experiment, care was taken to avoid growth of bacteria near the wall, through the use of special purifiers, and in addition, periodic replacement of the cultivator.

In another series of experiments, a method of short-term continuous experiments, in which a continuous culture is poured into several parallel cultivators with different propionate concentrations, which we developed (Neronova et al., 1966), was used to study the influence of propionate upon the bacterial growth rate.

A method of short-term experiments was used for the same purpose. A young culture (13–14 h), grown on a nonflowing medium, was poured into a series of flasks, and one amount of propionate or acetate or another was introduced into each of them. The products that were already accumulated in the culture before it was poured out were also taken into consideration. Immediately after pouring, as well as at the end of the experiment (which usually lasted for 1 h), the amount of bacterial biomass was determined

* Deceased.

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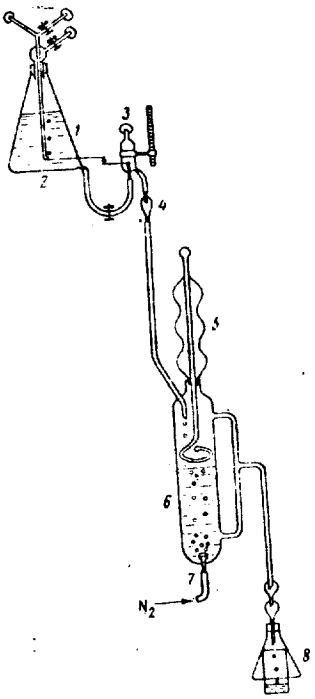

1. Scheme of apparatus continuous culturing of microorganisms. 1) Mariotte feed flask; 2) lower end of air tube in Mariotte flask; 3) regulating funnel; 4) drop counter; 5) cleaner of cultivator walls; 6) cultivator; 7) tube through which nitrogen is delivered; 8) receiver.

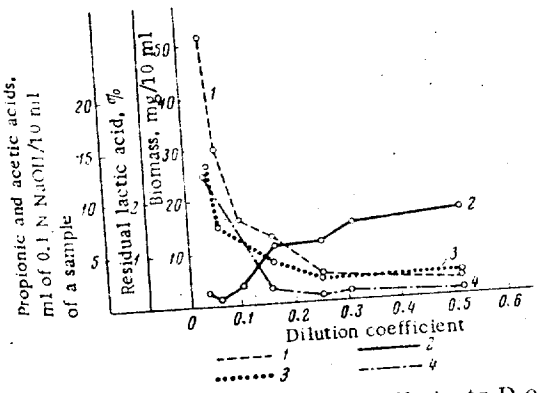

Fig. 3. Effect of the dilution coefficients D on the concentration of biomass, accululation of metabolic products (propionic and acetic acids) and on the specific growth rate of a continuous culture of P. shermanii. 1) Biomass; 2) lactic acid; 3) propionic acid; 4) acetic acid.

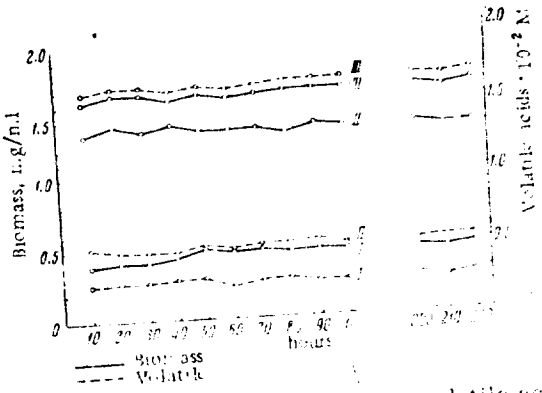

Fig. 2. Concentration of biomass in volatile acids at certain rates of flow during a stable state of a culture of P. shermanii. I) D = 0.25; II) D = 0.16; III) D = 0.10.

by nephelometry of the culture on the FEK-56. The bacterial growth rate coefficients were calculated on the basis of the data obtained.

RESULTS

As an example, Fig. 2 presents data on the concentration of biomass and volatile acids at certain rates of flow. The summary data on the biomass of the culture and concentrations of the components of the culture fluid at various rates of delivery of the medium are presented in the form of averages in Table 1 and Fig. 3.

The rates of flow are characterized in Fig. 3, by the dilution coefficients (D), i.e., by the ratio between the amount of the medium delivered per hour and the volume of liquid in the cultivator. As is well known, in the case of a stable state of the culture, the specific bacterial growth of rate (μ) is numerically equal to the dilution coefficient.

In our experiments, the pH was maintained at the same level. Residual lactate was always present in concentrations such as to insure the highest rate of growth of P. shermanii. Only in certain variations was its concentration too low, in view of which the specific rate of growth of the bacteria was converted to a lactate concentration of $12 \cdot 10^{-2}$ M according to the formula:

$$\mu_{12} = \frac{\mu (K_S + S_{12})}{(K_S + 12) S},$$

where μ is the actual rate of growth at a lactate concentration S and μ_{12} is the rate of growth at a lactate concentration $12 \cdot 10^{-2}$ M. The value of the constant K_S was taken from our work (Voronova et al., 1966).

TABLE 1. Dependence of the Growth Rate of P. shermanii on the Propionate Concentration during Prolonged Continuous Culturing

Dilution coeff., D	Initial lactate conc., 10^{-2} M	Residual lactate conc., 10^{-2} M	Specific growth rate converted to lactate, $12 \cdot 10^{-2}$ M	Biomass of culture fluid, mg/ml	Total amount of volatile acids, 10^{-2} M	Propionate conc., 10^{-2} M
0.04	29.3	2.66	0.062	5.10	25.5	12.4
0.06	21.1	1.44	0.128	2.94	18.2	8.2
0.10	21.1	3.77	0.137	1.59	16.1	7.6
0.16	21.1	11.7	0.16	0.31	4.5	3.2
0.25	21.1	12.0	0.25	0.46	1.9	2.2
0.32	21.1	15.9	0.35	0.39	1.9	1.3
0.54	21.1	17.9	0.56	0.36	1.7	1.1
1.0	19.4	19.1	1.00	0.08	—	0.09

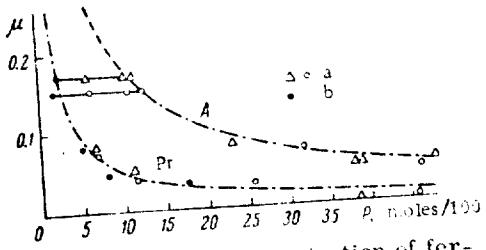

Fig. 4. Effect of concentration of fermentation products on the specific growth rate of P. shermanii according to the data of short-term experiments. A) Acetate added to the medium Pr) propionate added to the medium: a) in pure form; b) with admixture of acetate, 50% of the propionate.

It is distinctly evident (Table 1 and Fig. 3), that the decrease in the specific bacterial growth rate is paralleled by an increase in the content of metabolites in the medium (propionic and acetic acids). From this it may be concluded that the main factor determining the growth rate of P. shermanii is acids. In order to determine which of them more strongly inhibits the growth of the experimental culture, we used the method of short-term experiments described in the section "Procedure." The results are reflected in Fig. 4, from which it is evident that propionic acid has a stronger effect — it inhibits growth of the culture in substantially lower concentrations than acetic acid.

The relationship between the propionate concentration and bacterial growth rate is expressed by a gradually quenching bent curve. The curve of the relationship between the amount of added acetic acid and the growth rate of P. shermanii is of a different kind. Up to a concentration of $13-14 \cdot 10^{-2}$ M, acetate generally has no effect upon the bacterial growth rate, and only above this concentration does inhibition of growth begin.

Such a unique character of the curve is evidently explained by the fact that in the initial culture, after it has been poured out, there is always a certain amounts of propionic acid formed by the bacteria themselves (about $2-3 \cdot 10^{-2}$ M). Acetic acid added to the medium begins to exert an inhibiting effect upon the bacterial growth only after its amount is several times as great as the content of propionic acid, which corresponds to its lower activity. Thus, the effects of the two acids are not additive; the one of them that is present in a relatively large amount is active. To confirm this, we introduced various amounts of a mixture of propionate and acetate in a 1 : 2 ratio into the medium (Fig. 4). Just as we should have expected, the effect of this mixture of acids upon the growth rate of P. shermanii did not differ from the action of propionate alone. Under conditions of continuous culturing (Table 1 and Fig. 2), the amount of acetate, as a rule, was substantially smaller than that of propionate. Consequently, it may be considered that the principal active factor determining the rate of growth of a continuous culture of P. shermanii was propionate.

We used the method of short-term continuous experiments to refine this question.

In all the variations of the experiment, the residual lactate concentration and biomass concentration was maintained at the same level. The data obtained (Table 2) proved sufficiently close to what was established earlier (Table 1 and Fig. 2). Thus, it was finally confirmed that the main factor determining the

TABLE 2. Dependence of the Growth Rate of a Culture of P. shermanii on the Propionate Concentration According to the Data of Short-Term Continuous Experiments. Initial Lactate Concentration 11.4 · 10⁻² M

Dilution coeff., D	Propionic acid 10^{-2} M in medium delivered	Residual lactate concentration, 10^{-2} M	Biomass of culture fluid, mg/ml	Total amount of volatile acids, 10^{-2} M	Propionic acid concentration in culture, 10^{-2} M
0.648	0	1.07	0.10	0.97	0.76
0.413	1.15	1.09	0.11	2.23	1.4
0.274	2.49	1.19	0.13	3.36	2.75
0.176	5.30	1.17	0.12	6.49	5.4
0.104	11.2	1.13	0.12	12.10	11.2

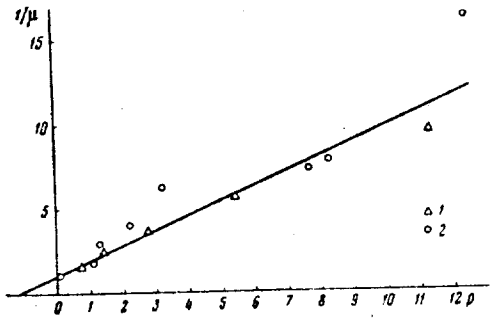

Fig. 5

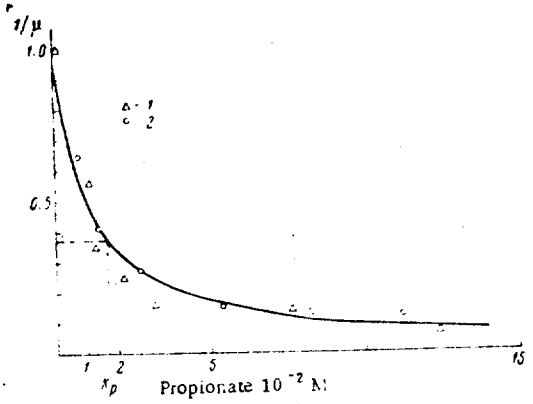

Fig. 6

Fig. 5. Determination of the constants graphically according to the Lineweaver-Berk method. 1) Data of short-term continuous experiments; 2) data of experiments with prolonged continuous culturing. (Results of two experiments, Tables 1 and 2).

Fig. 6. Relationship between the residual lactate concentration and specific growth rate of a continuous culture of P. shermanii. Each point is the average of two to four repeated experiments. Curve calculated according to the equation:

$$\mu = \frac{\mu_0 K_p}{K_p + P}, \quad \mu_0 = 0.95, \quad K_p = 1.1 \cdot 10^{-2}\ M$$

For notations see Fig. 5.

growth rate of a continuous culture of P. shermanii in these experiments is propionate. An analysis of the data obtained indicated (Ierusalimskii and Neronova, 1965), that the specific growth rate of the culture is related to the concentration of metabolites, in this case, propionate, by the following function:

$$\mu = \frac{\mu_0 K_p}{K_p + P},$$

where μ_0 is the specific growth rate on a definite nutrient medium in the total absence of growth inhibitors (propionate in the case considered); P is the concentration of this substance; K_p is a constant numerically equal to the propionate concentration at which

$$\mu = \frac{\mu_0}{2}.$$

The results of the two experiments (Tables 1 and 2) were combined, and on the basis of these data the values of μ_0 and K_p were determined graphically (Fig. 5) according to the method of Lineweaver and Berk (Yakovlev, 1964) $\mu_0 = 0.95$ and $K_p = 1.1 \cdot 10^{-2}$ M.

On the basis of the data obtained, we constructed a theoretical curve (Fig. 6), from which it is evident that the experimental data from the two experiments correspond well enough to the theoretical curve.

CONCLUSIONS

1. The method of continuous cultures and short-term experiments was used to determine the relationship between the concentration of fermentation products (propionic and acetic acids) and the growth rate of Propionibacterium shermanii.

2. It was shown that propionate and acetate may act as the growth rate controlling factors for a culture of these bacteria.

3. Acetic acid inhibits the growth of P. shermanii to a lesser extent than does propionic acid.

4. The inhibiting effects of the acids are not additive. When both acids are present in the medium, the growth rate of the culture is determined by the acid present in a relative concentration maximum.

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Volatile Chemical Compounds in Dry-Cured Hams[a,b]

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SUMMARY

Volatile compounds isolated from dry-cured hams were tentatively identified by gas chromatography retention times and further verification of the compounds made by infrared spectroscopy. These compounds were as follows: formaldehyde, acetaldehyde, propionaldehyde, isobutyraldehyde, *n*-valeraldehyde, isovaleraldehyde, acetone, diacetyl, methyl ethyl ketone, formic acid, acetic acid, propionic acid, butyric acid, and isocaproic acid. R_F values and selective indicators were used to identify ammonia and methylamine. Selective trapping was used to identify hydrogen sulfide and trace amounts of disulfides and/or monosulfides.

INTRODUCTION

The commercial economic importance of cured country-style hams (hereinafter referred to as CS hams) is increasing in the southeastern United States. This type of ham was described by Dunker and Hankins (1951).

The acceptability of CS hams is determined by odor and flavor, among other quality factors. A few studies have attempted to relate odor and or flavor to specific chemical constituents (Besley and Cecil, 1942; Blumer, 1954, 1958; Brady et al., 1949; Cecil and Woodroof, 1954; Pace and Barbella, 1937; Hunt *et al.*, 1939; Pace *et al.*, 1957, 1961).

Other studies of CS hams related to aroma and flavor have been conducted primarily by subjective methods. These methods may delineate the range of acceptability, but they are not precise enough to measure accurately the effect of treatments on the development of aroma and flavor. The aroma from CS hams is distinctive and resembles the flavor; therefore, it seemed reasonable to assume that the determination of volatile constituents is important for objective evaluation of these quality criteria. This study was initiated to determine methods satisfactory for the isolation, separation, and identification of volatile compounds in dry-cured CS hams under prescribed experimental conditions.

MATERIALS AND METHODS

Curing and aging of hams. Sixty-four regular short-cut skinned hams, weighing 14–16 lb, were cured for 2 days per lb at 4°C with 1 oz of curing mixture (8 lb NaCl, 2 lb white sucrose, and 3 oz potassium nitrate) per pound of ham. One-third of the total quantity required for each ham was applied to the surface on each of the 1st, 3rd, and 10th days. After curing, the hams were soaked 2 hr in cold water, dried, and then smoked at 21°C by burning hardwood sawdust. They were aged at 23±1°C, relative humidity 60±3%, and air flow 35 ft/min. Hams were sampled after they were cured and also after they were smoked. They were sampled during aging at monthly intervals for 6 months, and after 9, 12, 15, and 24 months. One ham was randomly selected for analysis at each of the sampling periods.

The NaCl and moisture content of hams will be quite uniform after curing 2 days/lb and storing for an additional 30 days.

Vacuum distillation. A 400-g portion of a 1-in.-thick cross-sectional center-cut ham slice was freed of subcutaneous fat, ground in a Waring blender and placed in a sample flask, and the volatile compounds were separated by vacuum dis-

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[b] This work is cooperative with the U. S. Department of Agriculture.

[c] From a portion of a Ph.D. thesis, North Carolina State College, 1962.

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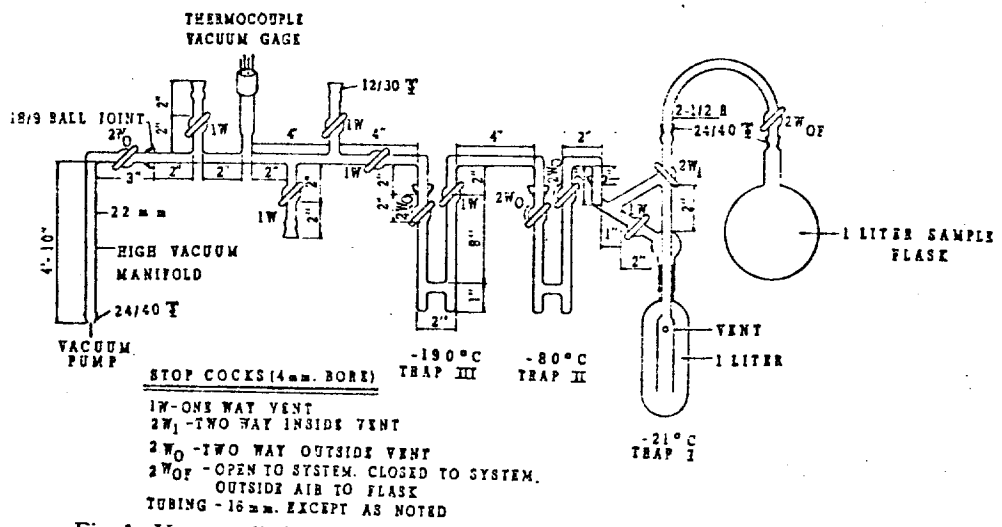

Fig. 1. Vacuum distillation apparatus (modification of Niegisch and Stahl, 1956).

tillation (Fig. 1). The sample was distilled 24 hr at 60°C under a vacuum of 200 μ. Typical aroma and flavor were developed under these conditions. Traps, arranged in order of decreasing temperature, were cooled with ice-salt, dry ice-methanol, and liquid nitrogen. After distillation, the contents of the traps were poured or bubbled into appropriate trapping solutions as described below. The odor after distillation was considered nearly neutral when the odor of the residue was compared with the odor of cooked portions of similar hams. Thus, it appeared that the distillation procedure for removal of volatile constituents was satisfactory. However, it is conceivable that higher distillation temperatures would produce compounds different from those produced here.

Trapping the volatile carbonyl compounds. Volatile carbonyl compounds were trapped in a solution containing 2 g 2,4-dinitrophenylhydrazine (DNP) per liter of 2*N* hydrochloric acid. The resulting 2,4-dinitrophenylhydrazones were collected by filtration and dried, first in air and then in a vacuum desiccator over phosphorus pentoxide.

Micro quantities of other carbonyl compounds, if present, may not have been detectable by the above technique.

Volatile carbonyl compounds from hardwood smoke were also trapped in DNP solution by pulling the smoke with a vacuum through a gas washing bottle containing the solution.

Gas chromatography of volatile carbonyl compounds. The carbonyl derivatives were analyzed by gas chromatography using a modification of the method of Ralls (1960a). An approximate 10-mg portion of a mixture of the hydrazones and alpha-ketoglutaric acid (1:3 by wt) was introduced into a Pyrex capillary tube sealed at one end, and prepared for injection into a gas chromatography (GC) apparatus (Fig. 2). The parent carbonyl compounds were regenerated and swept into the GC apparatus (Model 154-B Vapor Fractometer, Perkin-Elmer Corp.) by heating the capillary tube to 250°C in 15 sec and maintaining this temperature for an additional 15 sec.

The column (2 m × 6.35 mm) used to separate the carbonyl compounds was packed with Celite coated with diisodecyl phthalate, and maintained at 82°C. The carrier gas (helium) pressure in the column was regulated at 10 lb/sq in. and gave a flow rate of 32 ml/min (Woods, 1960).

To recover the separated carbonyl compounds, the tip of a glass tube attached to the exit port of the gas chromatograph was immersed in 5 ml of DNP solution whenever the recorder response indicated that a carbonyl compound was being eluted from the column. Several runs were made to obtain a sufficient quantity of hydrazone for subsequent identification by infrared spectroscopy. Each hydrazone was centrifuged from solution, washed with 2*N* HCl and then with distilled water, and dried over phosphorus pentoxide.

Infrared analysis of 2,4-dinitrophenylhydra-

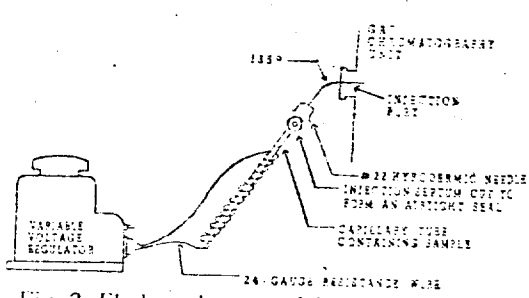

Fig. 2. Flash exchange and injection apparatus.

...es. The dried hydrazone was mixed with po...ium bromide (0.5:99.5) and placed in an ...uble potassium bromide die (Research and ...strial Instrument Co.). Vacuum was applied ...the die for 15 min, and the die, still under ...uum, was placed in a press (17,000 lb/sq in.) ... additional minutes. The pellet thus formed ... placed in the sample beam of a double beam ...del 137 Infracord Spectrophotometer (Perkin-...mer Corp.) and the spectrum recorded. The ...ctrum was compared with 2,4-dinitrophenyl-...drazones of known carbonyl compounds (East-...an Chemical Co.) purified by gas chromatography ... described above. Thus, known compounds from ...fferent homologous series could be differentiated ... well as compounds having different chemical ...oups.

Trapping the volatile acids. Short-chain volatile ...ids were trapped in 1*N* potassium hydroxide ...olution. The potassium salts thus formed were ...llected over a steam bath and dried in a vacuum ...en at 100°C.

Gas chromatography analysis of volatile acids. The method of Ralls (1960b) was used in the ... analysis of the volatile acids. The potassium ...lts of the acids were heated with potassium ethyl ...lfate to form the ethyl esters (Evans and ...Mertson, 1917). The esters were separated by ... procedure similar to that described above for ... carbonyl compounds, except that the injection ...illary temperature was raised to 300°C and the ...umn temperature to 120°C.

...s the individual ethyl esters emerged from the ... apparatus, they were trapped in a U-tube sub-...rged in liquid nitrogen. The esters were then ...rmed to room temperature and dissolved in car-... tetrachloride (CCl_4).

Infrared analysis of esters. An aliquot of a ...ution of each ester in CCl_4 (5:95) was placed ... a sodium chloride cell, and the infrared spectrum ...as recorded with CCl_4 the blank in the reference ...am.

Trapping of volatile basic compounds. The ...m samples were steam distilled at atmospheric ...ssure, and the volatile basic compounds were ...ed into a cold trap (ice and salt). The non-...ndensable gas was then bubbled through 1*N* ...Cl. The trapped components were combined and ...ed on a steam bath, and the drying was com-...ted in a vacuum oven at 100°C.

Paper chromatography of volatile basic compounds. The hydrochlorides of the volatile basic ...mpounds were separated by ascending paper ...hromatography by the method proposed by Da-...ies *et al.* (1953), as modified by Hornstein *et al.* ...1960) and Hornstein (1960). Whatman No. 1 paper was used as the stationary phase. The chro-...matogram was developed in a test tube (25 × 250 mm) for 90 min.

Selective trapping of volatile sulfur compounds. A slurry was made by blending 600 g of ham with 300 ml of distilled water. A sulfur absorption train as described by Dateo *et al.* (1957) and Hasselstrom (1957) was used to trap the volatile sulfur components. Refluxing and sweeping with nitrogen (30 ml/min) was continued for 5½ hr.

RESULTS AND DISCUSSION

Carbonyl compounds in hams. Fig. 3 is a representative chromatogram of the carbonyl compounds from one ham. The retention times for known carbonyl compounds are given in Table 1. From these values, the peaks of the chromatogram in Fig. 3 were tentatively identified.

For a more positive identification, the compounds labeled in Fig. 3 as acetaldehyde, propionaldehyde, isobutyraldehyde, diacetyl, 2-butanone (methyl ethyl ketone),

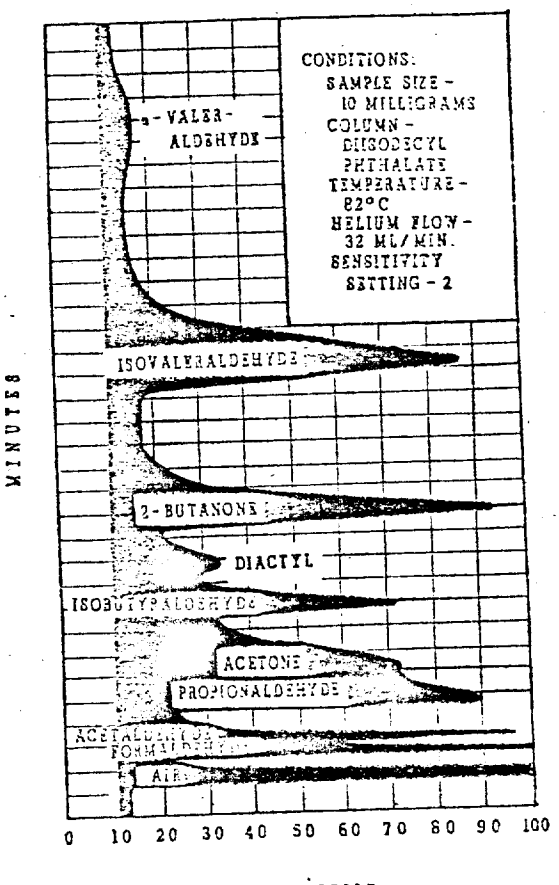

Fig. 3. Gas chromatogram of volatile carbonyl compounds isolated from a ham aged six months.

Table 1. Retention times of carbonyl compounds regenerated from 2,4-dinitrophenylhydrazones by exchange with alpha-ketoglutaric acid.

Parent carbonyl	Retention time (min) [a]	Retention volume (ml) [b]
Formaldehyde	1.4	45
Acetaldehyde	2.2	70
Propionaldehyde	4.0	128
Acrolein	4.2	134
Acetone	4.7	150
Isobutyraldehyde	7.7	246
n-Butyraldehyde	11.4	365
Diacetyl	11.5	368
Methyl ethyl ketone	11.8	378
Methyl isopropyl ketone	16.8	538
Isovaleraldehyde	17.8	570
Crotonaldehyde	20.5	656
Aldol	21.0	672
Methyl *n*-propyl ketone	21.5	688
Diethyl ketone	22.2	710
n-Valeraldehyde	25.7	822

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 82°C, and a helium flow of 32 ml/min.
[b] Retention volume = retention time × flow rate (32 ml/min.).

and isovaleraldehyde were trapped in the DNP solution as they emerged from the gas chromatograph, after which they were subjected to infrared spectroscopy. Excellent agreement was found between different classes of the known and sample compounds. However, the spectrum of a known sample of diacetyl was similar to the spectrum produced by a 75:25 mixture of diacetyl and isobutyraldehyde. It is therefore probable that the "diacetyl" peak of the sample contained some isobutyraldehyde. Other investigators have reported the following volatile carbonyls in meat: acetaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), Gaddis and Ellis (1959), Landmann (1960), Hornstein (1960), and Kramlich and Pearson (1960); propionaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), and Gaddis and Ellis (1959); diacetyl, Pettet and Lane (1940) and Pippen *et al.* (1958); and 2-butanone, Gaddis *et al.* (1959) and Pippen *et al.* (1958).

Attempts failed to trap the gas corresponding to formaldehyde (Fig. 3) and produce the hydrazone derivative. This result may have been due to the highly volatile nature of the gas.

Other carbonyl compounds listed in Fig. were found in trace amounts only; therefore, no further identification was made. However, acetone has been reported in pork fat by Gaddis *et al.* (1959), in chicken by Pippen *et al.* (1958), and in beef and pork by Landmann (1960) and Hornstein (1960). Callow (1927) identified formaldehyde in smoked meats, and Hornstein (1960) listed it as a constituent of beef.

Wood smoke was analyzed, and peaks were recorded with retention times characteristic of formaldehyde, acetaldehyde, acetone, methyl ethyl ketone, isovaleraldehyde, and *n*-valeraldehyde. Pettet and Lane (1940) also isolated formaldehyde, acetaldehyde, and acetone; however, they also found diacetyl, which was not identified with the procedure used here. Callow (1927) also found formaldehyde to be present in wood smoke.

The ratios among the carbonyl compounds as determined by gas chromatography remained quite constant during the entire aging period, except for 2-butanone. For this compound, the proportion appeared to increase during the latter part of the aging period. Although the ratios remained constant, the total quantity of carbonyl derivatives obtained from the hams increased with aging time. This agrees with the results of Pippen *et al.* (1958), Gaddis and Ellis (1957), Gaddis *et al.* (1959), Hornstein (1960), and Mendelsohn and Steinberg (1962), who found that conditions favorable to oxidation gave an increase in carbonyl compounds.

Whether or not a quantitative relationship exists between flavor and carbonyl compounds is not reported in this paper.

Volatile acids in hams. Fig. 4 is a representative chromatogram indicating the volatile acids (esters) obtained from a ham aged 9 months. These acids were identified by comparison with chromatograms of known acids, retention times for which are shown in Table 2. The acids found in the hams were tentatively identified as formic, acetic, propionic, butyric, and isocaproic.

Sufficient quantities of acetic and butyric acids were trapped to allow comparison of their infrared spectra with those of known

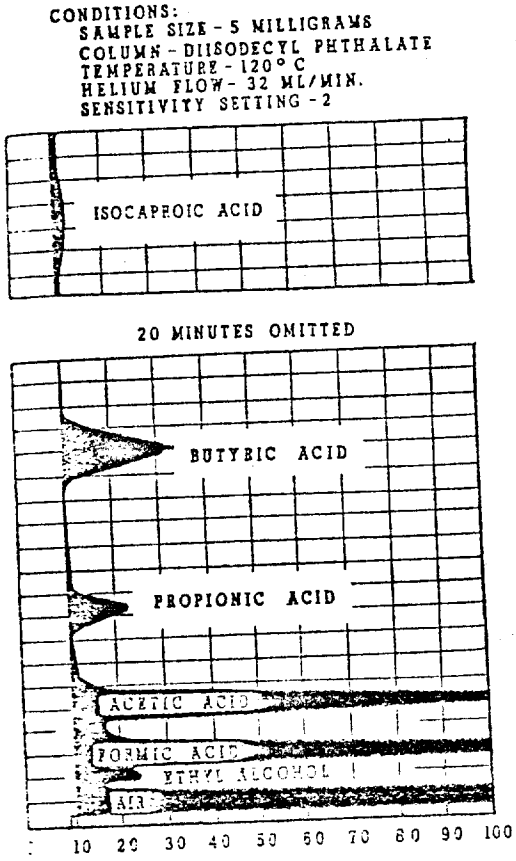

Fig. 4. Gas chromatogram of volatile acids isolated from a ham aged nine months.

...is. Excellent agreement was found; thus, ...fication of the same chemical grouping ... shown. Acetic acid was isolated from ... hams by Blumer (1954). The other ...s mentioned above could not be isolated ...quantities sufficient to form derivatives. ...ly one peak was obtained from the smoke ...lysis having a retention time the same as ... of the formic ester.

The number and quantity of acids iso... were found to increase with the length ... the aging period, except for the quantity ... formic acid. The percentage of this acid ...reased with storage time, but the relative ...crease was probably due to an increase of ...nger-chain acids rather than to a decrease ... amount of formic acid. It should be men...ioned at this point, however, that formic ...cid is a constituent of wood smoke, and ...some decrease of the acid from this source may have occurred, especially since the residual of wood smoke is deposited primarily upon the meat surface.

Table 2. Retention times for ethyl esters of volatile acids.

Parent acid	Retention time (min) [a]	Retention volume (ml) [b]
Ethyl alcohol [c]	1.9	61
Formic	2.4	77
Acetic	4.3	138
Acrylic	7.7	246
Propionic	7.9	253
Isobutyric	10.3	330
Trimethylacetic (pivalic)	11.8	378
Methacrylic	12.5	400
Butyric	14.2	454
Isovaleric	20.4	653
Crotonic	21.5	688
Valeric	27.5	880
Dimethylacrylic	36.7	1174
Isocaproic	39.7	1270
Caproic	53.3	1706

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 120°C, and a helium flow of 32 ml/min.

[b] Retention volume = retention time × flow rate (ml/min).

[c] Ethyl alcohol is sometimes a disintegration product of the ester formation, thus forming a peak on the gas chromatography tracing.

Volatile bases in hams. Table 3 shows the paper chromatography results for known amine hydrochlorides. Comparison of these values with those obtained from the spots on the chromatogram from the ham samples indicates that the major portion of the volatile bases was ammonia and a very small amount was methylamine. This is in agreement with the results reported by Hornstein *et al.* (1960) and Hornstein (1960) for beef, and with the basic components of meat flavor as listed by Landmann (1960); however, Landmann also noted the presence of ethylamine, which was not detected in this study. Pippen and Eyring (1957) found only ammonia in chicken broth.

Volatile sulfur compounds in hams. Almost immediately upon refluxing and sweeping with nitrogen, lead sulfide precipitate was noted in the solid lead acetate trap, indicating that hydrogen sulfide had been liberated from the ham under these conditions. Several research workers have reported the presence of hydrogen sulfide in extracts of

Table 3. Paper chromatography separation of known amine hydrochlorides.

Compound	R_F	Ninhydrin test	Methyl orange test
NH_4Cl	0.50	Negative	Positive
$CH_3NH_2 \cdot HCl$	0.45	Positive	Positive
$CH_3CH_2NH_2 \cdot HCl$	0.53	Positive	Positive
$CH_3(CH_2)_2NH_2 \cdot HCl$	0.60	Positive	Positive
$CH_3(CH_2)_3NH_2 \cdot HCl$	0.68	Positive	Positive
$(CH_3CH_2)_2NH \cdot HCl$	0.78	Negative	Positive

meats (Crocker, 1948; Bouthilet, 1951a,b; Pippen and Eyring, 1957; Hornstein, 1960). A 3½-hr period of continuous refluxing followed before a trace of other precipitates was noted in the aqueous mercuric chloride traps. Thus, with a longer refluxing time, a trace of disulfides and/or monosulfides was obtained. Under the conditions of this experiment no mercaptans were detected.

The methods of analysis used in this study appear to be, in general, adequate for determining the volatile compounds in CS hams. Since these hams were aged for long periods, some differences in the amount and kind of these compounds were noted at the several sampling periods. Some association may exist between aged flavor and the development of certain of these compounds. In future quantitative work dealing with flavor of CS hams, the possibility should be taken into consideration that carbonyl compounds do make some contribution to the flavor complex.

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The influence of ruminal infusion of volatile fatty acids on milk yield and composition and on energy utilization by lactating cows

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1. In an experiment of 3 × 3 latin square design, four lactating Holstein cows were given a basal ration designed to induce low percentages of milk fat. The treatments were (1) basal ration, a pelleted mixture of lucerne hay (20%) and concentrates (80%), with 40 l. of water infused intraruminally, (2) basal ration with acetic acid substituted for 15·4% of the metabolizable energy (ME) and (3) propionic acid substituted for 15·4% of the ME. In the last 3 weeks of the 6-week experimental period respiration trials were carried out in an open-circuit indirect calorimeter. The levels of feeding offered in the three periods were 325, 275 and 225 kcal ME/kg body-weight $^{0.75}$ in periods 1, 2 and 3 respectively.

2. No differences were detected in the utilization of the energy of acetic and propionic acids, but there were differences in the partition of energy into milk or body tissues; with acetic acid infusion more energy was secreted as milk and with propionic acid infusion more was deposited in body tissue.

3. There was an increase in milk fat percentage with acetic acid infusion, but not complete recovery to normal. The milk fat percentages were 1·96, 2·58 and 1·92 for treatments 1, 2 and 3 respectively. Acetic acid infusion caused increases in the C12, C14 and C16 fatty acids of milk fat and decreased the proportion of C18:1 fatty acids.

4. It is suggested that the low percentages of milk fat found when cows are given concentrates could result from a decreased extent of fermentation in the rumen, allowing a greater proportion of the starch consumed to be absorbed as glucose in the small intestine.

The efficiency of utilization of volatile fatty acids (VFA) for different physiological functions has been studied by several methods. Differences in the proportions of acetic, propionic and butyric acids in VFA mixtures continuously infused intraruminally into fasting sheep had only a small effect on the efficiency of energy utilization (Armstrong, Blaxter & Graham, 1957). For fattening, all acids were utilized less efficiently than for maintenance, particularly acetic acid (Armstrong & Blaxter, 1957) and mixtures containing a high proportion of acetic acid (Armstrong, Blaxter, Graham & Wainman, 1958). With salts of VFA, however, no difference could be found between acetate, propionate and butyrate in the ability to promote growth in young sheep (Orskov & Allen, 1966*a*, *b*, *c*; Orskov, Hovell & Allen, 1966). In lactating goats supplementation of a basal ration with a mixture of VFA or with acetic or propionic acid (Armstrong & Blaxter, 1965) had only a minor effect on energy utilization, as judged by the heat increment.

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The effect of VFA on the percentage and composition of milk fat has been extensively studied (Stoddard, Allen & Peterson, 1949; Tyznik & Allen, 1951; Balch, Balch, Bartlett, Cox & Rowland, 1952; Balch & Rowland, 1959; Shaw, Robinson, Senger, Lakshmanan & Lewis, 1959; Rook & Balch, 1961; Stanley, Morita & Ueyama, 1964; Rook, Balch & Johnson, 1965; Jorgensen, Schultz & Barr, 1965; Storry & Rook, 1966) and although the results vary in magnitude it has been established that increases in milk-fat percentage occur when diets which induce low milk-fat percentage are supplemented with acetic acid or sodium acetate (Tyznik & Allen, 1951; Balch & Rowland 1959; Rook & Balch, 1961; Stanley *et al.* 1964; Rook *et al.* 1965; Jorgensen *et al.* 1965; Storry & Rook, 1966). However, a complete recovery of milk-fat percentage on such rations has seldom been accomplished. Recently Storry & Rook (1966) showed that intraruminal infusion of acetic acid caused milk fat to recover to an extent approximating to only about 25% of the decrease, indicating that factors other than acetic acid are involved. The depression of milk-fat percentage generally arose from a greater reduction of the C4–C16 acids (Balch *et al.* 1952; Balch & Rowland, 1959; Shaw *et al.* 1959; Storry & Rook, 1966) than of the C18 acids, and various suggestions have been made to explain the phenomenon.

In the experiment reported here the effects of acetic and propionic acid infusion on milk-fat percentage and composition were studied. In addition calorimetric measurements were obtained which allowed some assessment of the efficiency with which the energy of the infused acids was utilized. While it would have been desirable to have used two different basal rations, the amount of work involved prevented this and it was decided to use a basal ration which was expected to induce low milk-fat percentage to ensure that the milk fat might be influenced by the treatment imposed.

MATERIALS AND METHODS

Animals. Four lactating Holstein cows, in which rumen fistulas had been established 3 months before the experimental period, were used. The calving dates, body-weights, ages and other relevant information are given in Table 1.

Table 1. *Information about the experimental cows*

Cow no.	Date of birth	Last calving date	No. of lactation	Weeks of lactation on experiment	Body-wt (kg)	Breeding date
456	15 Sept. 1962	6 July 1965	1	38–55	571	Not pregnant
459	7 Nov. 1962	24 Dec. 1965	2	14–31	553	1 Sept. 1966
445	17 Mar. 1966	7 Mar. 1966	2	7–23	653	Not pregnant
465	4 Jan. 1963	16 Dec. 1966	2	17–35	584	5 Apr. 1966

Design and treatments. A 3 × 3 latin square design was used; an additional animal (465) was also used which received the same treatment sequence as one of the cows in the 3 × 3 design.

To achieve a completely balanced design, the animals should be of the same age and stage of lactation. It was not possible to obtain cows with established rumen fistulas

which conformed to this requirement, but all animals were in a stage of lactation where calving dates or pregnancy were not likely to influence milk composition; three cows were in mid-lactation and cow 456, which was in late lactation, was not pregnant.

The treatments were (1) basal ration plus 40 l. of water, (2) basal ration plus 40 l. of acetic acid solution and (3) basal ration plus 40 l. of propionic acid solution. The water and acid solutions were infused intraruminally. The length of the periods was 6 weeks, of which 3 weeks were preliminary; calorimetric measurements were obtained during the last 3 weeks of each period.

Diets. In period 1 the level of feeding was calculated to supply 325 kcal metabolizable energy/kg body-weight$^{0.75}$ (ME/kg$^{0.75}$), in period 2 this was 275 kcal, and in period 3, 225 kcal ME/kg$^{0.75}$. The basal ration was for this purpose estimated to contain 2·72 kcal ME/g and the ME of the VFA was assumed to be the heat of combustion (Hodgman, 1962) of the acids. In the periods when acids were infused the acids were calculated to replace 15·4% of the ME of the ration. The amounts of acetic and propionic acids infused were for period 1, 1633 and 1149 g/day, for period 2, 1492 and 1149 g/day, and for period 3, 1155 and 901 g/day respectively.

The basal ration was a pelleted mixture of 20% finely ground (1·6 mm screen) lucerne hay (*Medicago sativa*) and 80% concentrate. The ingredients of the concentrate were maize meal 73·75%, soya-bean meal 24·25%, trace-mineralized salt 1·00% and steamed-bone meal 1·00%. The average chemical composition of the pelleted ration was, as percentage of dry matter, nitrogen 3·12, carbon 44·98, ether extract 2·05, ash 5·09, acid detergent fibre (ADF) 12·89, lignin 2·23 and neutral detergent fibre (NDF) 25·20, and its calorific value was 4·45 kcal/g. Acetic acid was found to contain 3·35 and propionic acid 4·87 kcal/g.

Fatty acid infusion. The daily infusion of acids was usually given in 23 h to allow time for the animals to exercise while the chambers were open and while the tanks were refilled. Slow speed pumps (Sigmamotor) were used to regulate the rate of infusion both in the preliminary period and when the cows were in respiration chambers. Acid-resistant Tygon tubing was inserted into the rumen through the cannula with about 60 cm inside positioned so that the acids were not flowing directly on to the rumen wall.

Management of the animals. The cows were milked twice daily. Uneaten food was collected once daily and samples were obtained for determination of dry matter and chemical analysis. During the preliminary periods the animals were allowed 1 h exercise daily in a concrete yard. During the periods in the respiration chambers the animals were given exercise, and body-weights were recorded on days when the chambers were not sealed. Body temperature, heart rate measurements and checks on udder condition and general health were made every day in the energy metabolism laboratory. The animals were given the basal diet in two equal feeds at 07·00 h and 17·00 h.

Collection of foods, faeces, urine, milk, blood and rumen fluid. During each period complete collections and analyses of foods, uneaten foods, faeces, milk and urine were made during two 5-day periods and one 7-day period. The samples were bulked during these periods and preserved for analysis by freezing or drying. On the last day of the 7-day

period, at 11·00 h, samples of rumen fluid were obtained via the fistula and samples of blood from the mammary vein. The rumen samples were preserved with 2% saturated $HgCl_2$ and the blood with NaF.

Respiration trials. Open-circuit respiration chambers were used for collection and determination of carbon dioxide, methane and oxygen as described by Flatt, Van Soest, Sykes & Moore (1958) and Moe & Flatt (1967). The chambers were sealed at the end of the 2nd day after collections of urine and faeces had begun and remained sealed for 5 consecutive days. Measurements in the respiration chamber were then taken for the last 2 days of period 1 and the first 3 days of period 2. After that a third collection period was started within which faeces and urine were collected for 7 days and respiration measurements were made on 5 days, giving a total of 17 days collection of urine and faeces and ten 24 h respiration trials. Small subsamples of incoming air and exhaust gas from the chambers were collected in spirometers continuously over 24 h periods and analysed daily. The results were automatically recorded on punch cards by the system described by Moe & Flatt (1967). Total heat production was calculated from the factors adopted at the Third Symposium on Energy Metabolism (Brouwer, 1965). The chambers were kept at an average temperature of 17·7° and a relative humidity of 58%.

Analytical methods. The methods for analysis for nitrogen, ash, ether extract and dry matter were the standard procedures adopted by the Association of Official Agricultural Chemists (1960). Lignin, ADF and NDF were analysed by the method of Van Soest (1963*a*, 1965). Gross energy was determined in a Parr adiabatic bomb calorimeter with an automatic temperature controller (Arthur H. Thomas Co., Philadelphia, USA). The carbon determinations were made with an induction furnace and gasometric analyser as modified and described by Smith, Flatt, Barnes & Van Soest (1965). Milk yields, corrected for solids content, to achieve equal energy/unit of milk were calculated according to Tyrrell & Reid (1965). Milk fat was determined by the Babcock test, solids-not-fat (SNF) by lactometer, and milk protein by the Orange G dye test (Udy, 1956). Ketone body estimations were made by the method of Behre (1940). For the respiration trials, carbon dioxide and methane were analysed in Beckman Model LB 15 infrared analysers and oxygen in a Beckman Model G 2 paramagnetic analyser. Determinations of pH in rumen fluid were made immediately after sampling and straining with a Beckman Zeromatic glass electrode pH meter. VFA determinations were made on strained rumen fluid, after acidification with 25% metaphosphoric acid (5 ml of rumen fluid to 1 ml of acid), by gas–liquid chromatography with a flame ionization detector.

The milk fat was separated by centrifugation. Methyl esters of the fatty acids were prepared by adding approximately 50 mg of fat to 1 ml of 1% (v/v) H_2SO_4 in methanol. Methylene chloride (about 0·5 ml) was then added until the fat was completely dissolved. The container was tightly capped and allowed to stand at room temperature for 14 h. After the addition of 2 ml water, the esters were extracted into two 3 ml portions of hexane. The esters were recovered after evaporating the hexane on a steam bath under a slow stream of nitrogen. The methyl esters were subjected to gas chromatography on a 6 ft × 4 mm stainless steel column packed with diethyleneglycol

succinate polyester (20%) on 80–90 mesh Anakron ABS. The esters were separated at 170° with an inlet pressure of 0·703 kg/cm². The peak areas were measured by triangulation.

Statistical methods. In periods 2 and 3 statistical analyses were performed on the over-all means for the periods totalling 17 days of faeces and urine collection and 10 days of respiration measurements. In period 1 the first 5 days respiration measurements for cows 456 and 459 were discarded because of technical failures; the intake of food and the energy balance were calculated from the last 7-day period of faeces and urine collection and the last 5 days of calorimetric measurements. During period 3, cow 445 became ill and lost appetite, consequently a missing plot had to be calculated which, in the statistical analyses, resulted in only 1 degree of freedom for error. In all the statistical analyses, therefore, the between-cow variability has been pooled into the error term. The standard error of means is the weighted mean standard error for comparing means with two or three observations. The regression analyses (Fig. 1), however, are based on each individual collection period, including those for the cow which was not in the regular design, and totalled twenty-eight collection periods.

RESULTS

In Table 2 the food offered and consumed and the excretion of dry matter and ADF are given. The food left uneaten was slightly greater when VFA was infused, though the differences were small and not significant. During two trials in period 2, cows 459 and 456 were given the uneaten food via their rumen fistulas. This procedure was

Table 2. *Daily food offered and consumed, volatile fatty acids (VFA) infused, faecal excretion of dry matter (DM) and acid detergent fibre (ADF), and daily intake of metabolizable energy (ME)*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of ME replaced by acetic or propionic acid)

Treatment	Basal ration offered (kg)	VFA (g)	Basal ration consumed (kg)	Faecal excretion of DM (kg)	Faecal excretion of ADF (kg)	ME intake (Mcal)
Basal	11·22	0	10·70	2·83	1·03	31·30
Basal + acetic acid	8·90	1427	8·12	2·07	0·72	29·18
Basal + propionic acid	9·12	1043	8·21	1·95	0·65	30·10
SE of means	—	—	0·43	0·22	0·06	0·43

Table 3. *Intake of energy and loss of energy (Mcal/day) in faeces, urine, methane and heat lost in milk*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of metabolizable energy replaced by acetic or propionic acid)

Treatment	Gross energy intake	Faecal output	Urine output	Methane production	Heat production	Milk energy	Body tissue energy balance
Basal	47·63	13·25	1·73	1·33	17·23	10·47	+3·62
Basal + acetic acid	40·85	9·65	1·25	0·83	16·20	9·42	+3·41
Basal + propionic acid	41·82	8·30	1·45	1·10	17·14	7·56	+6·27
SE of means	0·50	0·87	0·54	0·88	0·71	0·71	0·52

discontinued when it was found that progressively more food was left uneaten. The infusion of VFA did not seem to influence the digestibility of the basal ration, as the apparent digestibility of dietary dry matter (excluding infused VFA) was almost the same with each treatment.

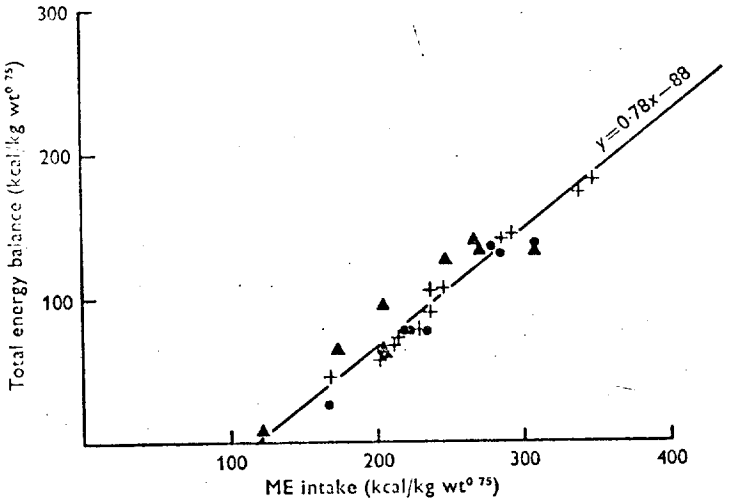

Fig. 1. Relation between energy balance (milk + body tissue) and intake of metabolizable energy (ME) for cows receiving a basal ration (+) or having 15·4% of ME replaced by acetic acid (▲) or propionic acid (●).

Table 4. *Intake of nitrogen and nitrogen loss in faeces, urine and milk (g/day)*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of metabolizable energy replaced by acetic or propionic acid)

Treatment	Nitrogen intake	Faecal nitrogen	Urinary nitrogen	Milk nitrogen	Nitrogen balance
Basal	333	96	116	101	+20
Basal + acetic acid	253	69	91	69	+14
Basal + propionic acid	234	65	65	72	+32
SE of means	11	5	4	14	13

Energy balance. Table 3 summarizes the energy balance of the animals. There were differences in the gross energy intake partly because of uneaten food but mainly because the infused VFA were assumed to be 100% digestible and substituted on a ME basis. The differences in faecal energy were similar in magnitude to the differences in faecal dry-matter excretion. The reduction in energy losses as urine and methane when VFA were infused, although not statistically significant, reflect the decreased intake of fermentable feed. There were no significant differences in heat production. The energy secreted as milk, expressed as a percentage of intake, was greater when acetic acid was infused than with propionic acid infusion, whereas in tissue energy balance the reverse was found. In Fig. 1 the ME intakes expressed as kcal/kg$^{0.75}$ have been plotted against total energy balance (milk + body tissue), also expressed as kcal/kg$^{0.75}$.

Table 5. *Yields of milk and solids-corrected milk (SCM), yields and content of milk fat, solids-not-fat (SNF) and protein and milk energy content*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of metabolizable energy replaced with acetic or propionic acid)

Treatment	Milk yield (kg/day)	SCM (kg/day)	Milk fat		SNF		Protein		Energy (kcal/g)
			g/day	%	kg/day	%	g/day	%	
Basal	19·19	13·91	357	1·96	1·67	8·64	612	3·19	0·55
Basal + acetic acid	15·58	12·18	376	2·58	1·33	8·53	489	3·15	0·61
Basal + propionic acid	14·65	10·13	254	1·92	1·24	8·35	450	3·05	0·54
SE of means	2·58	1·18	21	0·33	0·20	0·23	90	0·09	0·03

Table 6. *Composition of the fatty acid fraction of the milk fat (g/100 g total fatty acids), assuming that the C4 to C10 acids constitute 10% of the total*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of metabolizable energy replaced by acetic or propionic acid)

Treatment	C12:0	C14:0	C14:1 and C15:0	C16:0	C16:1	C18:0	C18:1	C18:2
Basal	1·75	9·83	4·11	26·60	2·63	4·28	35·11	5·54
Basal + acetic acid	2·29	13·13	3·21	34·82	3·61	3·32	25·95	3·68
Basal + propionic acid	3·25	12·37	2·88	28·08	2·42	5·68	30·96	4·33
SE of means	0·17	1·29	0·27	2·14	0·62	0·71	2·32	1·36
Fasting (single value)	0·70	2·50	1·40	24·90	3·40	8·30	46·10	2·70

Table 7. *The pH, total volatile fatty acid (VFA) concentration and molar percentages of VFA in rumen fluid, and the concentration of ketone bodies in blood drawn from the mammary vein*

(Mean values are given for three cows receiving a pelleted basal ration or having 15·4% of metabolizable energy replaced by acetic or propionic acid)

Treatment	Rumen fluid								Blood ketone bodies (mg acetone/100 ml)
	pH	VFA (m-equiv./l.)	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	
Basal	5·52	123·2	54·4	28·5	1·6	11·6	1·5	2·3	8·3
Basal + acetic acid	5·67	143·6	70·8	17·4	8·7	0·7	1·5	1·5	12·4
Basal + propionic acid	5·36	88·5	49·6	44·0	1·0	3·0	1·4	1·2	6·6
SE of means	0·41	30·6	4·4	3·2	0·3	2·7	0·7	0·7	1·1

There was a highly significant ($P < 0.001$) linear relationship, $y = 0.78x - 88$, where y = total energy balance, expressed as kcal/kg$^{0.75}$ and x = ME intake in kcal/kg$^{0.75}$. The standard error of the regression coefficient was ± 0.04 and the correlation coefficient $r = 0.96$.

Nitrogen balance. The differences in nitrogen intake (Table 4) reflect differences in intake of the basal ration. The urinary excretion of nitrogen was greater when acetic acid was infused than when propionic acid was infused so more nitrogen was retained in the latter treatment, though not significantly so.

Milk yield and composition. In Table 5 the mean values for milk yield and composition have been summarized. The yield of milk was highest when the basal ration was fed. Between the VFA treatments the yields were slightly, but not significantly, greater when acetic acid was infused. This difference was accentuated when expressed as solids-corrected milk because the milk-fat percentage was highest when acetic acid was infused. There were no differences in milk-fat percentage between the basal ration and the propionic acid treatments. The energy content of the milk reflected differences in the milk-fat percentage.

Milk fat composition. The milk fat composition is given in Table 6. Increases in the C12:0 and C14:0 fractions occurred with both acetic and propionic acid infusions. The most noticeable differences were in the increases in the C16:0 and concomitant decreases in the C18:1 fraction when acetic acid was infused. During period 3, when cow 445 was taken off experiment, a fasting metabolism trial was conducted with her and the milk fat compositions have been included after 5 days of fasting.

VFA composition. Table 7 shows the concentration and proportions of VFA in the rumen liquor and the concentration of ketones in the blood. As would be expected, there were large increases in the proportions and concentrations of the acids that were infused. The proportion of butyric acid on the propionic acid treatment was very low, resulting in the production of a negative value for the missing plot of cow 445 in period 3. Blood ketone concentration was greatest when acetic acid was infused.

DISCUSSION

The experiment reported has obvious shortcomings, mainly due to the small number of animals studied and to the difficulty of constructing a design which would be completely balanced. These limitations were partly due to the amount of work involved in respiration trials and also to the difficulty of having cows successfully fistulated. The results therefore must be considered as a first modest contribution towards solving a very complex problem in the nutrition of the dairy cow; conclusions reached in the following discussion might well be modified when more information of this type becomes available.

Effect of VFA on energy utilization. No difference in energy utilization by the cows could be detected between the treatments, and there was no suggestion of a lower efficiency of utilization of acetic acid than of the metabolizable energy of the basal ration or of propionic acid. This is illustrated in Fig. 1, where the energy balance has been plotted against the metabolizable energy intakes per unit of the body-weight$^{0.75}$.

The figure includes the values for cow 465 and shows the mean values in each of the twenty-eight collection periods. Despite the fact that the molar proportion of acetic acid in the rumen content varied from 50% when propionic acid was infused to 70% when acetic acid was infused, no difference in the slopes was evident. This suggests that differences in heat production between rations for dairy cows were not associated with an inefficient utilization of acetic acid and is in agreement with results obtained with fattening lambs (Orskov & Allen 1966*a*, *b*, *c*; Ørskov *et al.* 1966). Though there was no difference in energy utilization between the treatments, there were apparent differences in the partition of dietary energy into milk energy or body storage. The ratio of milk energy to ME intake was higher when acetic acid was infused than when propionic acid was infused, 0·33 as opposed to 0·26, a difference which was statistically significant ($P < 0·05$). Rook & Balch (1961) found that milk yield increased when acetic acid, but not when propionic acid, was infused.

These trends are similar to those observed in another experiment conducted at Beltsville (Flatt, Moe, Moore, Hooven, Lehman, Hemken & Orskov, 1967; Flatt, Moe, Munson & Cooper, 1967) which showed that diets giving rise to a high proportion of propionic acid in the rumen resulted in a greater deposition of tissue energy than diets in which a high proportion of acetic acid was found, although intakes of metabolizable energy were similar.

The changes in blood ketone concentrations were in the direction that might be expected if acetic acid was exerting an apparent ketogenic effect. However, no clinical signs of ketosis were noted in this experiment.

Effect of VFA on the fat content of milk. The effects noted on fat content of milk show a similar trend to those observed by other workers (Tyznik & Allen, 1951; Balch & Rowland, 1959; Rook & Balch, 1961; Stanley *et al.* 1964; Jorgensen *et al.* 1965; Rook *et al.* 1965; Storry & Rook, 1966). This demonstrates that if the molar proportions of the VFA in the rumen fluid on a diet inducing milk of low fat content were changed by infusion to attain molar proportions which are not normally associated with such milk, the fat content is still not raised to normal levels (Storry & Rook, 1966). It has been suggested (Van Soest, 1963*b*) that digestion and absorption of starch in the small intestine may be as effective in depressing the fat content of cow's milk as production and absorption of propionic acid in the rumen. If this theory is extended to suggest that it is the gluconeogenic energy ratio (defined as the energy absorbed as acetic acid and butyric acids in relation to that absorbed as propionic acid and glucose) which is most important, an interpretation of the results may be possible. Recently, experiments have shown that substantial quantities of starch can escape rumen fermentation (Wright, Grainger & Marco, 1966; Karr, Little & Mitchell, 1966). In recent experiments at Beltsville we have used a fermentation balance approach to estimate factors influencing extent of fermentation (Orskov, Flatt & Moe, 1968). This approach requires calculation, from the VFA proportions found in the rumen, of the amount of methane that would be produced if all digestible carbohydrate were fermented. The calculated methane production was then related to the determined methane production and used as a guide to estimate the extent of fermentation. It was then found that increased level of feeding, increased proportions of concentrate and

decreased particle size all tended to decrease the extent of fermentation and that, with the basal ration used here, about 50% of the digestible carbohydrate escaped fermentation; this observation is in agreement with the results of Karr *et al.* (1966).

If this approach is valid, our results suggest that a recovery of milk-fat percentage could not be expected by restoring the molar proportions of VFA to proportions not normally associated with low milk fat content. This is because the molar proportions of VFA do not represent the same proportions of dietary energy as when the proportions of VFA are measured on, for instance, a hay diet; in that instance the calculated extent of fermentation was much greater (Orskov *et al.* 1968). The results also indicate that the proportion of the diet digested in the small intestine and absorbed, presumably as glucose, could be a very important contributor to the glucose pool. It is thus possible that any factors which reduce the rate of fermentation or increase the rate of passage of concentrate diets (Van Soest, 1963*b*) are likely to decrease the yield of milk fat while factors which have the converse effects will increase it. The effects of dietary supplements of cod-liver oil (Shaw & Ensor, 1959) and bicarbonate (Emery, Brown & Bell, 1965) on milk fat might well be associated with effects on rate of fermentation. Infusion of propionic acid here did not further depress the fat content of the milk, suggesting a limit to which it can be depressed by these means. The mode of action is likely to be associated with the influence of glucogenic materials on the plasma glyceride level as shown by McClymont & Vallance (1962).

Influence on milk-fat composition. The influence of VFA infusion on the composition of milk fat was similar to that which has been reported previously by other workers (Balch *et al.* 1952; Balch & Rowland, 1959; Storry & Rook, 1966). During fasting the milk-fat composition approached that of body fat.

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Chronicity of Acetic Acid Ulcer in the Rat Stomach

Susumu Okabe, PhD and Carl J. Pfeiffer, PhD

Experimental gastric ulcers, produced by submucosal injection of acetic acid in rats were studied in relationship to healing. Small ulcers healed completely within 40 days after induction; moderately severe ulcers were sometimes found at 150 days, and probably resulted from repeated healing and reulceration. Large severe ulcers partially healed and then became reexacerbated; they frequently increased in size up to 250 days, thus resembling chronic gastric lesions. Throughout the experimental periods, adhesion of the ulcer base with adjacent organs and delayed gastric emptying were found in most animals. After ulceration, an increase in gastric volume and acid output and a decrease in protease activity of gastric contents were found in chronic fistula rats with experimental ulcers. The secretory changes observed here may have been secondary to gastric ulceration and/or delayed gastric emptying. The factors responsible for the reexacerbation of the experimental ulcer remain unidentified.

In contrast to human peptic ulcer disease, deep or superficial experimental gastric or duodenal ulcers in laboratory animals usually heal within 3 to 4 weeks after induction. Such experimental lesions reportedly do not break down and reulcerate spontaneously under a normal feeding schedule. Additional treatments, such as the administration of ulcerogenic agents (1–4), artificial pyloric obstruction (5, 6) or surgical procedures (7, 8), can aggravate the lesions or delay healing, but healing eventually occurs when treatment is discontinued. Hence, these may not be appropriate models of human chronic ulcer, and the question posed over a hundred years ago, "Why do acute ulcers in the human often become chronic?" remains unsolved.

In 1969, Takagi et al (9) reported that an experimental ulcer in rats, produced by submucosal injection of acetic acid, persisted for at least 150 days. More recently, the present authors found in preliminary studies that this experimental ulcer, after partial healing at 60 days, was still apparent even at 200 days (10), thus resembling the chronicity of human peptic ulcer. In order to clarify the mechanisms of this chronicity, detailed investigations on the healing processes in relation to size, location and adhesion to adjacent organs were undertaken up to a maximum of 250 days after ulcer induction. In addition, basal gastric secretion of acid and pepsin, before and after experimental ulceration, were determined in chronic fistula rats.

MATERIALS AND METHODS

Gastric Ulceration

As previously reported (10, 11), male Sprague-Dawley rats weighing 240 to 270 g were used. During other stud-

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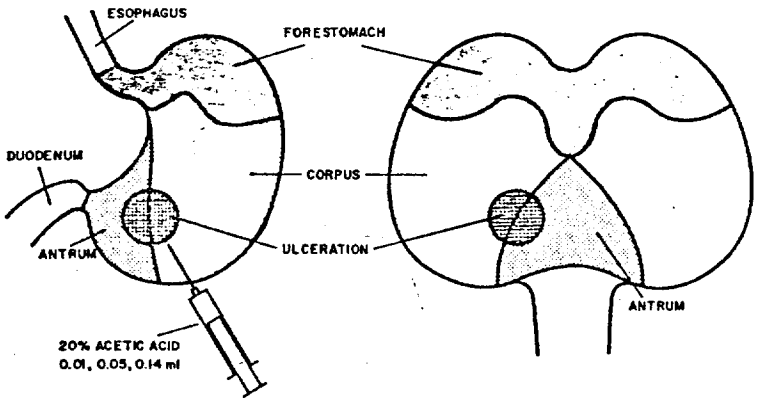

Fig 1. Schematic drawing of rat stomach, indicating site of experimental ulcer.

thesia, a midline, epigastric laparotomy was made in the rats, and after exteriorizing the stomach, 0.01, 0.05 or 0.14 ml of 20% acetic acid was injected into the submucosal layer of the anterior or posterior wall of the glandular stomach (Figure 1). After injection, the abdomen was closed and all rats were maintained normally on Purina Laboratory Chow and water ad lib. Animals were sacrificed at 5, 10, 20, 40, 60, 80, 100, 150, 200 or 250 days after the operation, to assess the healing process of the ulcer. Each stomach was removed, filled with 10 to 15 ml of 1% formalin, and immersed in 1% formalin for 5 minutes to fix the outer layer of the gastric wall (12). Subsequently, the stomach was opened along the greater curvature, examined grossly for lesions, and the length and width of the lesions were measured. The products of length times width were graded according to the ulcer index shown in Table 1. Adhesion of the base of the ulcer to the surrounding organs (mainly liver with anterior ulcer, and pancreas and omental fat with posterior ulcer) was observed in the majority of animals.

Table 1. Indices of Lesioned and Adhered Areas of Gastric Ulcers

Products of length and width of lesioned area (sq mm)	Ulcer index	Products of length and width of adhered area (sq mm)	Adhesion index
0	0	0	0
1–8	1	1–10	1
9–24	2	11–30	2
25–63	3	31–100	3
64–120	4	101–200	4
121–168	5	>201	5
>169	6	—	—

Therefore, the products of the measured width and length of the adhered regions were rated to assess degree of adhesion, according to the adhesion index shown in Table 1. Specimens were then fixed in 10% neutral, buffered formalin and were studied histologically after staining with hematoxylin and eosin.

Gastric Secretion

Stainless steel cannulae (11 mm length, 4 mm ID) were implanted in the forestomach of 36 male Sprague-Dawley rats (400 to 470 g) according to the method of Alphin and Lin (13). At least 2 weeks were allowed for recovery after surgery prior to gastric function tests, and rats were not tested more often than once a week. Prior to secretory tests, individual rats were fasted 24 hours (water ad lib); they were then put into nonrestraining shoebox-type cages for the collection of gastric juice. The stomach was washed several times via the gastric cannula with 0.9% saline and allowed to drain for 30 minutes before each test.

Gastric contents were then collected for 3 hours (10 AM to 1 PM) in graduated test tubes during basal secretion, and volume output, titratable acidity (titrated electrometrically to pH 7.4 with 0.025 N NaOH) and acid output were determined for each sample. In addition, total protease activity (pepsin plus gastricsin) was determined (Beckman DU-2 Spectrophotometer) on 0.5 ml samples of filtrated gastric juice by the method of Chiang et al (14), using a 2% bovine hemoglobin substrate. Animals were randomly divided into two groups for these collections, and basal secretion tests were replicated 3 times (control tests) for all rats. Subsequently, one group was subjected to laparotomy alone (sham control), and experimental gastric ulcers were induced in the other group by injection of 20% acetic acid (0.14 ml) into the anterior wall, as described above. Four of 25 animals subjected to experimental ulceration succumbed to free perforation of the gastric ulcer at 2 to 3 days after

operation. Gastric secretory tests were continued for up to 15 weeks (105 days) after operation, and animals were then sacrificed by ether and ulcer size was determined.

RESULTS

The healing processes of experimental gastric ulcers of different size and location are delineated in Figures 2 and 3. The degree of adhesion between the ulcer base and the surrounding organs is illustrated in Figure 4.

Healing of anterior gastric ulcers produced by 0.01 ml of 20% acetic acid. Typically, a small ulcer was found on the anterior mucosal wall 5 days after operation. The ulcer histologically penetrated the entire gastric wall; in most cases it healed completely within 40 days and never re-ulcerated during observations up to 100 days (Figure 2). Fibrous adhesion with the adjacent liver was observed in more than 60% of animals throughout the entire experimental period, although the adhered area was small and limited to the corpus. Thus, it can be assumed that the ulcer was completely healed, although the adhesion was still present.

Healing of anterior gastric ulcer produced by 0.05 ml of 20% acetic acid. Relatively large, clearly demarcated ulcers were found in all animals on the fifth day after operation, and the ulcer floor was extensively covered with necrotic debris. Adhesion of the ulcer base with the liver, induced by confined perforation, was moderately extensive and persisted throughout the 150-day experimental period. These ulcers quickly diminished in size and depth until 20 to 40 days by granulation from the base and epithelization from the edge of the ulcer. Thereafter, some of the animals showed complete healing of the ulcer, but others had small lesions in the mucosa at 60, 80, 100 or 150 days after ulcer induction. At 100 days, 75% (9/12) of the rats had discrete ulcers (Figure 2), probably resulting from the breakdown of previously induced but healed lesions. In general, animals having a clearly defined ulcer showed gastric retention, since the stomach was filled with food residue. The margins of the lesions were typically indurated, and acute or chronic gastritis was frequently observed on the area adjacent to the chronic ulcer.

Healing of posterior gastric ulcers produced by 0.05 ml of 20% acetic acid. Mortality rates attributable to unconfined gastric perforation after ulcer preparation in the posterior wall were higher (10 to 15%) than in the anterior ulcer groups (7%). At autopsy, the outstanding feature of the stomach a few days after the operation was marked gastric retention due to food accumulation. Although the initial size and depth of posterior ulcers and anterior ulcers were almost the same at the time of ulceration, large posterior ulcers were still found in many instances at 40 or 60 days. At 150 days, more than 80% of rats had a small ulcer and, in rats with such lesions, there invariably was concomitant, severe gastric dilatation with food residue. The ulcer base was strongly adhered to the pancreas and/or omental fat, and the degree of adhesion was almost the same throughout the duration of experiments.

Healing of anterior gastric ulcers produced by 0.14 ml of 20% acetic acid. At 5 days, large and deeply demarcated penetrating ulcers were seen in the anterior wall, thus presenting a high average ulcer index of 5 (Figure 3). Mortality rates due to gastric perforation were about 15 to 20%. Rapid healing was evident after ulceration, as with 0.01 and 0.05 ml-treated rats at 20 to 40 days. At 60 days, 12 of 20 animals showed complete healing—ie, newly formed epithelia covered the entire surface of the defect. Other animals also revealed a greatly diminished defect size, although 1 animal had a large gastric ulcer. At 80 days, 70% of animals showed complete healing. During the 100- to 200-day period after operation, more than 43% of rats had discrete ulcers, with ulcer indices varying from 6 to 1. Hyperplasia of the gastric

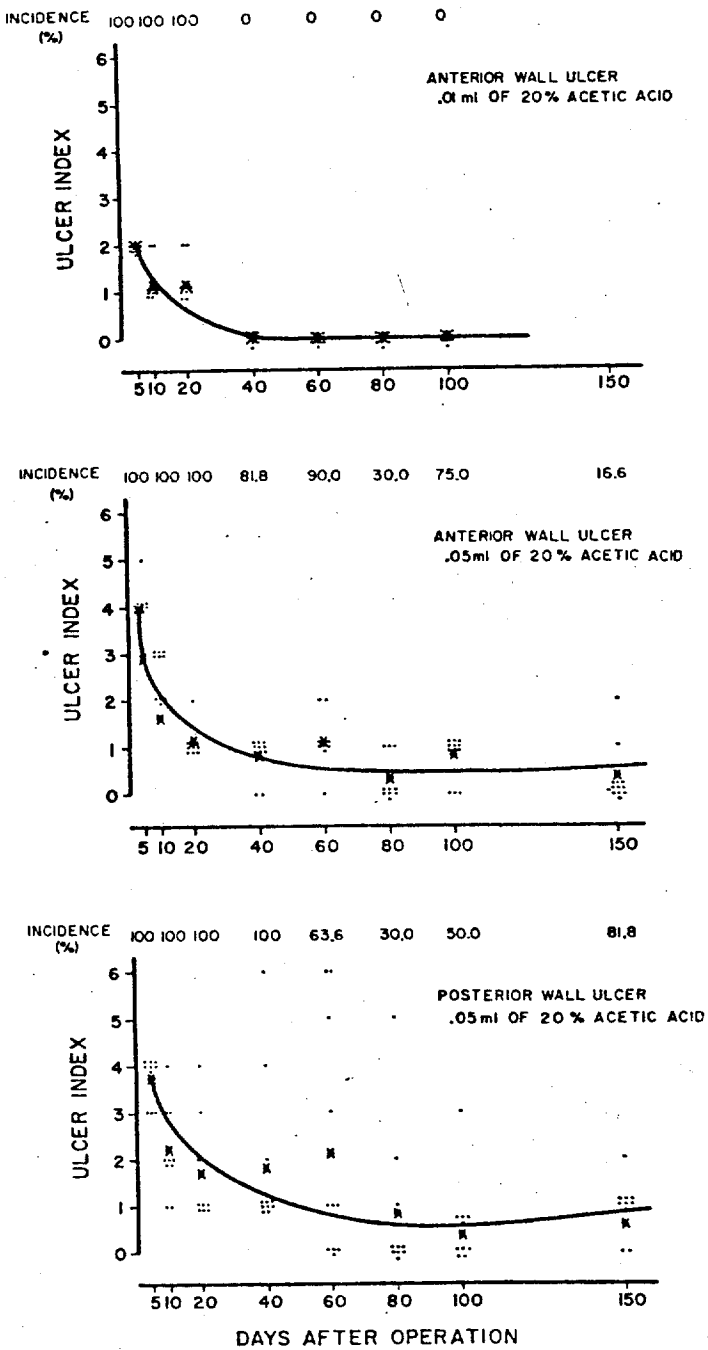

Fig 2. Healing of gastric ulcers produced by submucosal injection of acetic acid into the anterior or posterior wall of the rat stomach. **Large dots** represent the mean ulcer index and **small dots** represent values of individual rats.

ACETIC ACID ULCER IN RAT STOMACH

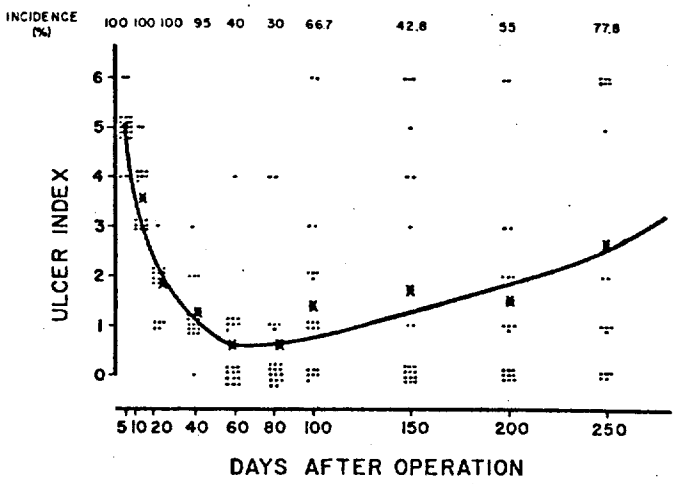

Fig 3. Healing of gastric ulcers produced by submucosal injection of 20% acetic acid (.14 ml) into the anterior wall of the rat stomach. Note the elevation of the curve with the passing of time, indicating the reexacerbation of healed or partially healed ulcers. **Large dots** represent the mean ulcer index and **small dots** represent the values of individual rats.

mucosa was frequently found in stomachs with ulcers. Minor mucosal defects on the surface of completely or partially healed ulcers were observed frequently, suggesting the beginning of recurrence. These minor lesions were usually situated off-center of the position of the originally ulcerated area. At 250 days, 6 of 18 animals had large ulcers (25 × 22, 23 × 20, 18 × 18, 18 × 14, 14 × 13, 13 × 13 mm in size), which were larger than or similar to the initial lesions; such lesions had extensive inflammatory exudates on the floor of the ulcer (Figure 5).

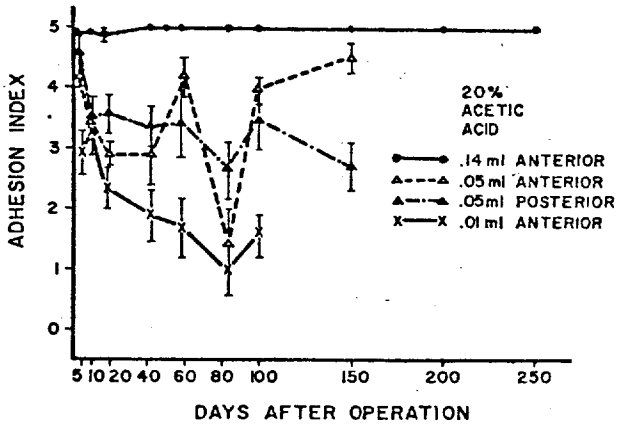

Fig 4. Degree of adhesion of the base of the experimental ulcer with the adjacent organs after ulcer induction.

Fig 5. Gross appearance of a gastric ulcer produced by submucosal injection of .14 ml of 20% acetic acid into the anterior wall of the rat stomach (Day 250). Note the large size of the lesion and the extensive debris on the floor of the ulcer.

In view of the size and time sequence of these lesions, this ulcer model might realistically be called a progressive chronic ulcer. Adhesions in these lesions involved the anterior wall of the pylorus and duodenum, as well as the base of the ulcer, as a result of confined perforation in the majority of animals.

Gastric distention, presumably resulting from impaired gastric emptying, was observed throughout the experiment. Gastric contents were not quantitatively measured, however. Large ulcers were invariably associated with greatly distended stomachs.

Healing rates of anterior and posterior ulcers. The healing rates (mm/day) of anterior and posterior wall ulcers are summarized in Table 2. The healing rates varied, depending upon size and location of the ulcer, and almost invariably diminished with time. During the 5-day period from the fifth to tenth day after operation, the mucosal healing rate of the moderate or large ulcers in the anterior or posterior gastric wall was significantly higher than the rate with small lesions. The healing rate of posterior ulcers became significantly slower than that of the anterior ulcer, produced with the same volume of acetic acid, during the 10- to 20-day period. At the 20- to 40-day interval, the healing rates of the above four groups became similar.

Basal gastric secretion during healing of the acetic acid ulcer. Experiments were carried out for 105 days after ulcer preparation, an apparent minimal time for healing and reulceration of the acetic acid ulcer. At 1 week after the preparation of the lesion, several animals (8/21) demonstrated notable bleeding from the stomach during the collection of gastric juice, apparently induced by the mechanical irritation of the new ulcer by the lavage procedure. Three of these 8 bleeding rats died, presumably from hemorrhage, within 1 or 2 days after the first gastric collection, and no free perforations were found. Subsequent gastric collections did not provide evidence of bleeding during the experiment. No control animals (sham-operated) bled. The weekly changes in basal gastric secretion of animals which were subjected to laparotomy or to experimental ulceration are delineated in Figure 6. Volume output and acid output were elevated significantly just after ulceration ($P < 0.01$, and 0.05, respectively). In the control group, volume output was essentially similar throughout the experimental period, but acid output was slightly increased after ulcer preparation. Titratable acidity remained unchanged in both groups, except on Day 14 and 28, at which times the nonulcerated group showed higher values. Protease activity, however, was markedly suppressed just after ulcer induction ($P < 0.001$) and remained low throughout the experiments.

At the conclusion of the experiments it was found that 7 of the 14 ulcer-treated rats had a discrete ulcer still remaining on the anterior gastric wall, with ulcer indices ranging from 1 to 4. The remainder showed complete healing

Table 2. Healing Rates of Acetic Acid Ulcers of Various Sizes and Sites in the Rat Stomach

	Treatment with 20% acetic acid				
	A 0.01 ml anterior wall	B 0.05 ml anterior wall	C 0.05 ml posterior wall	D 0.14 ml anterior wall	*P*
Diameter (maximum width of initial size) at Day 5 (mm)	4.70 ± 0.21 (10)	9.90 ± 0.38 (10)	9.00 ± 0.61 (10)	13.85 ± 0.41 (20)	A:B <0.001 A:C <0.001 A:D <0.001 B:C NS B:D <0.001 C:D <0.001
Healing rate (mm/day)					
Period I (Day 5–10)	0.40 ± 0.06 (10)	0.78 ± 0.09 (10)	0.91 ± 0.13 (10)	0.85 ± 0.14 (18)	A:B <0.001 A:C <0.001 A:D <0.01 B:C NS B:D NS C:D NS
Period II (Day 10–20)	0.09 ± 0.03 (10)	0.34 ± 0.02 (10)	0.07 ± 0.09 (10)	0.56 ± 0.03 (18)	A:B <0.001 A:C NS A:D <0.001 B:C <0.01 B:D <0.001 C:D <0.001
Period III (Day 20–40)	0.09 (10)	0.07 ± 0.01 (11)	0.07 ± 0.05 (10)	0.11 ± 0.01 (20)	B:C NS B:D <0.05 C:D NS
P	I:II <0.001	I:II <0.001 I:III <0.001 II:III <0.001	I:II <0.001 I:III <0.001 II:III NS	I:II <0.01 I:III <0.001 II:III <0.001	

Values are mean ± SE
Numbers inside parentheses = no. of animals used
NS = not significant ($P > 0.05$)

with newly formed epithelium covering the defects. Stomachs of the control (laparotomy) animals revealed normal gastric mucosae. Accordingly, data from the experimental ulcer group were divided into two categories: healed and unhealed groups. Results of this subdivision are shown in Figure 7. Volume output and acid output of the unhealed group were statistically lower during the latter period of the experiment. However, the titratable acidity remained unchanged in both groups, and the decrease of protease activity was similar in both groups throughout the experiment.

DISCUSSION

Following production of ulcers in rats with topical acetic acid, the possibility of recurrence of previously healed ulcers was suggested in previous reports (9, 10), but only preliminary evidence existed. Results of the present experiments, based upon large numbers of animals and a larger experimental lesion, suggest, however, that ulcers observed after 100 days are probably re-ulcerated lesions. Specifically, with the present ulcer model (eg, induction by 0.14 ml of 20% acetic acid) all lesions rapidly and markedly decreased in size without exception

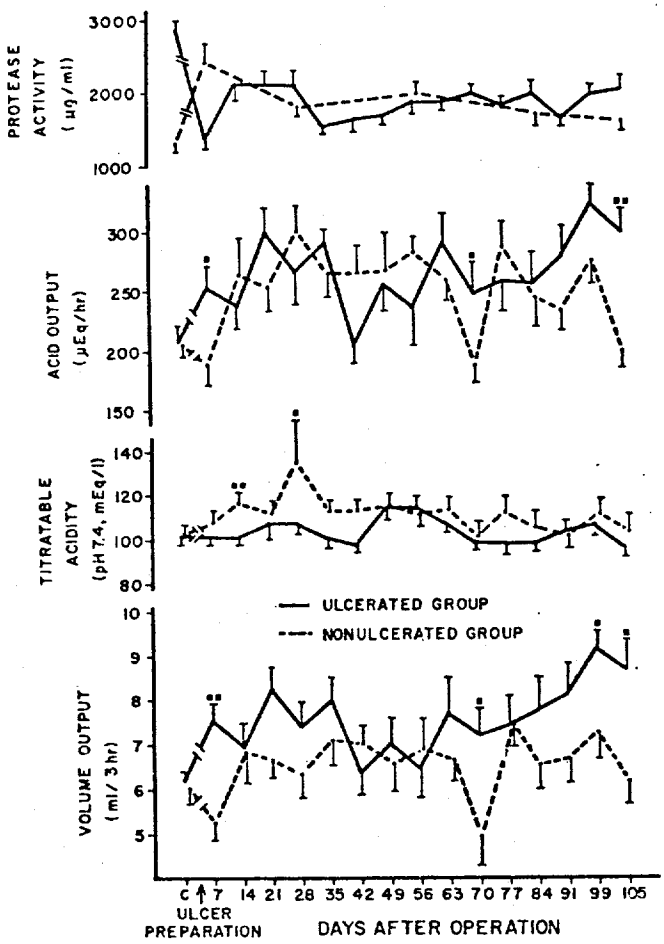

Fig 6. Gastric secretory changes in chronic fistula rats subjected to laparotomy alone or ulceration by acetic acid injection. Fourteen animals were used in each group; each **point** represents mean ± SE (Values significantly different from controls: ■ = $P<.05$. ■■ = $P<.01$. ■■■ = $P<.001$).

until 20 to 40 days after ulcer induction, and then enlarged.

Healing rates were rapid (0.1 mm/day) with large ulcers, and were in agreement with values from the literature dealing with the healing process of acute ulcers that healed completely (15). In contrast, the smaller ulcers elicited a slower healing rate but completely healed at 40 days. The reason for these differences in healing rates remains unclear.

One of the unique observations in this study was the enlargement of the ulcerated region in the latter phase of tissue repair. In particular, at Day 250 the incidence of ulcers reached a maximum 79%, and the ulcers in 6 of 18 animals were in size equal to or larger than the initial lesions. These observations suggest that the ulcers not only redeveloped but also increased in severity with time, although free perforations were not found. Posterior wall ulcers, in contrast to anterior wall ulcers, enlarged earlier (prior to Day 40), suggesting that "aggravative factors" appeared faster on the posterior gastric wall. The following factors can be considered in regard to the mechanisms of chronicity.

Adhesion of the base of ulcer to adjacent organs. Haubrich and co-workers (16–18)

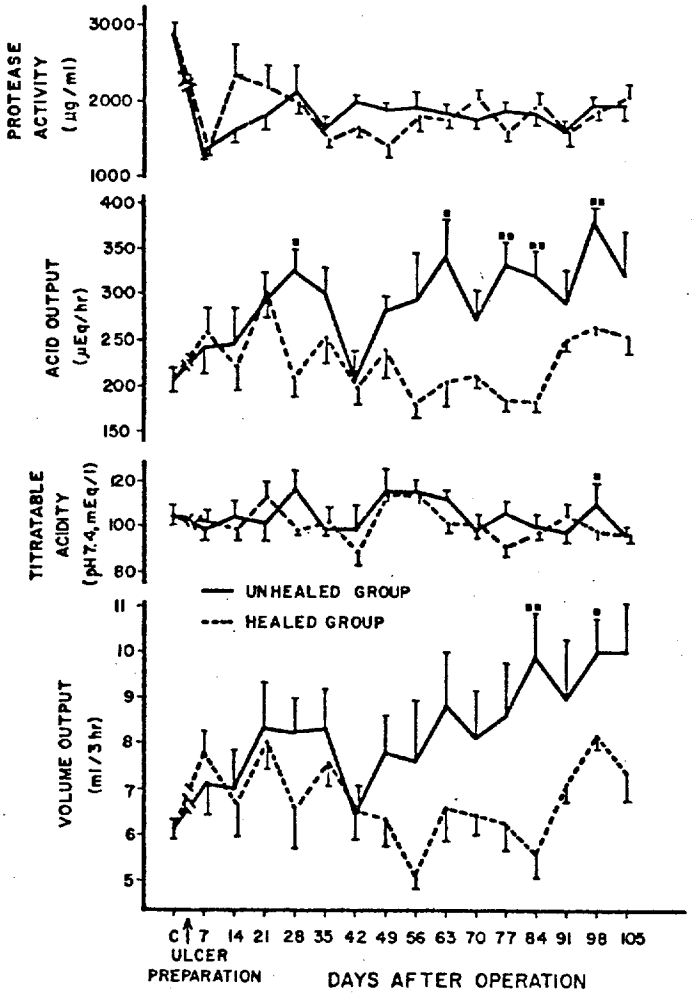

Fig 7. Gastric secretory changes in chronic fistula rats having healed or unhealed ulcers at the time of autopsy. Seven animals were used for each group and each **point** represents the mean ± SE (Values significantly different from control: ■ = $P < 0.05$, ■■ = $P < 0.01$).

have emphasized the fact that in human peptic ulcer disease, confined perforations of fibrous adhesions in many instances form the basis of "intractability" to the most rigid medical management. One of the prominent features of the experimental, acetic acid-induced ulcer is the adhesion of the base of ulcer to the liver, pancreas or omental fat, caused by penetration of the ulcer into these organs (19). There exists, therefore, a possibility that such adhesions might influence the healing process in some way. In the present experimental study it was observed that the adhered area between the ulcer base and the liver was almost invariably extended to the proximal duodenal wall, including the pyloric sphincter. It is possible that the observed gastric retention might have been caused by pyloric obstruction due to these adhesions.

Delayed emptying and gastric retention were also recognized with the posterior wall ulcer, the base of which was adhered to the pancreas and omental fat.

As previously mentioned, the small ulcer produced by injection of 0.01 ml of acetic acid healed without reexacerbation; the adhesion was slight and did not extend to the pyloric sphincter, and delayed emptying was not found.

Dragstedt and co-workers have suggested that gastric stasis or impaired gastric motility, with resultant hypersecretion, might be causal to the pathogenesis and chronicity of peptic ulcer (20, 21). This theory was partly based upon clinical experience with vagotomy in which stasis, produced by vagotomy alone without drainage procedure, sometimes resulted in the development of gastric ulceration or recurrence. It has been reported that bilateral, subdiaphragmatic vagotomy alone evoked a delayed healing of experimental acetic acid ulcer (9). Therefore, gastric stasis produced by adhesion might be a contributing factor to the chronicity of acetic acid ulcer. Furthermore, the food of laboratory rats is considerably more coarse than that of man, cats or dogs. Therefore, the mechanical trauma of rat chow upon the ulcerated mucosa and associated gastric stasis might be considered as one of the factors related to long persistence of ulcerative lesions, as suggested by Beazell and Ivy (22) and Ivy and Fauley (23).

Gastric secretion. It is well known that gastric secretion in gastric ulcer patients is either normal or reduced, compared to that observed in nonulcerated, healthy persons.

In rats ulcerated by acetic acid, the volume and acid output were steadily increased relative to sham-operated animals. The increases were especially marked in animals which had ulcers at the time of autopsy (Day 105), in contrast to those which had completely healed ulcers. In man and animals, it has been confirmed that pyloric obstruction or gastric retention, whatever the cause might be, evokes a vagovagal reflex and, or release of gastrin (24–27), or hyperplasia of parietal and peptic cells (28–30) with a resultant gastric hypersecretion. Therefore, the steady increase of gastric secretion following ulceration in the unhealed ulcer group might be attributable to chronic pyloric obstruction, as manifested by delayed emptying which was observed grossly. In contrast, the mechanism of decreased peptic activity is not known. Vagne and Grossman have reported that exogenous gastrin administered during gastric retention depressed pepsin secretion, while vagal stimulation always stimulated pepsin secretion (31). It is possible, therefore, that the retention caused by adhesion may have induced gastrin release from the antrum, with a resultant increase in acid output and decrease in peptic activity.

ACKNOWLEDGMENTS

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Pharmacological Effects of Fatty Acids, Triolein and Cottonseed Oil.

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Various triglycerides in emulsion show marked pharmacological properties on intravenous administration. For example, several with low molecular weights have been found distinctly toxic on intravenous injection (WRETLIND 1957 b; ORÖ, WESTERHOLM & WRETLIND 1959). On the other hand, fats of higher molecular weights, such as cottonseed oil, triolein and certain vegetable fats, show little or no toxicity on intravenous injection into mice. Hence it is possible to produce injectable emulsions of cottonseed oil as a carrier of agents, such as higher fatty acids, that owing to insolubility could not otherwise be studied for their pharmacological effects. Such fatty acid emulsions have enabled us to determine the toxicity of fatty acids on intravenous injection.

In the same investigation we have also studied the effects of these emulsions on respiration and blood pressure in cats. It was found that emulsions containing only cottonseed oil or triolein could cause reduced blood-pressure and apnoea. These effects were characterised by such pronounced tachyphylaxis that frequently they were noticeable only on the first injection. They were readily distinguishable, therefore, from the affects of the different fatty acid emulsions in which cottonseed oil acted as carrier.

Lastly, we conducted experiments on guinea pig small intestine in order to study the effects of fatty acid emulsions on smooth muscle.

Experimental.

Animal Experiments.

For *determinations of toxicity*, mice weighing 13–34 g were used. The emulsions or solutions of fatty acids were injected into the tail vein. Each fatty acid was administered to at least six groups each of 10 animals. The calculations of LD50 with their standard

errors were by the method of MILLER & TAINTER (1944); the results are summarised in figure 1.

Some 100 cats weighing 2-6 kg were used for investigating the *respiratory* and *circulatory* responses to cottonseed oil, triolein and fatty acid emulsions. These animals were anaesthetized with diallymal (allobarbital (WHO), dial ®, Ciba, 25–40 mg per kg body weight) or chloralose (50 mg/kg). Respiration and blood pressure were recorded by means of a Grass electroencephalograph[1]) with supplementary demodulators. A Statham pressure transducer, model P 23 AA[2]), was used for blood-pressure determinations on the carotid artery. The respiration was recorded with a Grass pressure transducer PT-5 connected to a side tube in a tracheal cannula.

In a number of experiments the *pulmonary arterial pressure* was measured via a plastic catheter that had been sutured in the artery after opening the thorax. In one experiment the thorax was closed again; in the others it was left open under artificial respiration.

For studying the direct effect of fatty acids upon the heart, a *heart-lung preparation* described by WRETLIND (1957 b) was used. Blood pressure and flow were recorded by the Grass transducers mentioned above.

The action of fatty acids on guinea pig *small intestine* was studied in an isolated organ bath containing 15 ml Tyrode solution (0.8 % NaCl; 0.02 % KCl; 0.02 % $CaCl_2$; 0.02 % $MgCl_2 \cdot 6\,H_2O$; 0.1 % $NaHCO_3$; 0.005 % $NaH_2PO_4 \cdot H_2O$; and 0.1 % glucose). The organ bath was aerated with air. A Grass force-displacement transducer, model FT-03, was employed for recording the tension of the gut. In this connection we also studied the ability of fatty acids to abolish contractions elicited by histamine. For this purpose histamine (0.2 µg/15 ml) was first added to the organ bath and fatty acid emulsion was added without prior washing when maximal contraction had been obtained. The observed relaxation of the intestine was recorded.

Test Solutions and Test Emulsions.

For the *toxicity determinations* in mice the lower *fatty acids* (acetic, propionic, butyric, isovaleric, norvaleric, caproic and heptylic) were administered in aqueous solution, whereas the higher fatty acids, because of their insolubility in water, were emulsified. To prepare these *emulsions* the fatty acids were first dissolved in heated cottonseed oil, which served as carrier. Phosphatides, sodium cholate and glycerin monostearate were used as emulsifiers (table 1). The emulsions were homogenized in a Logeman apparatus (WRETLIND 1957 a). The pH of the solutions and emulsions was adjusted to 7.3 with NaOH.

The concentrations of fatty acids were so chosen that the injected volume required for determining LD50 amounted to between 3 and 45 ml per kg body weight. Thus acetic, propionic, butyric, isovaleric, norvaleric and caproic acids were administered as 10 % and heptylic acid as 5 % aqueous solutions; caprylic, nonanoic, capric, undecylic, lauric, tridecylic and myristic acids as 2 % emulsions; pentadecylic, palmitic, margaric and stearic acids as 0.2 %, and oleic acid as 2 % emulsions.

For investigating the action of fatty acids on *guinea-pig gut*, 1 % emulsions of the compositions recorded in table 1 were used.

Cottonseed oil was similarly utilized as carrier when investigating the *respiratory* and *blood-pressure* responses of the cat to higher fatty acids. Emulsification was with phosphatides and Pluronic F 68 (table 2), because they make stabler emulsions of

1) Grass Instrument Co., Quincy, Mass., U.S.A.
2) Statham Instruments Inc., 254 Carpenter Road, Hato Rey, Puerto Rico.

Table 1.

Composition of fatty acid emulsions used for determining the toxicity in mice.

The cottonseed oil, supplied by Wesson Oil and Snowdrift Sales Co., New Orleans, La., U.S.A., was used after filtration at +4° C. The phosphatides were prepared as described WRETLIND (1957 a). The emulsions were adjusted to pH 7.3 with N-NaOH.

Fatty acid	0.2–2 g
Cottonseed oil	10 g
Phosphatides	0.4 g
Sodium cholate	0.1 g
Glycerol monostearate	0.5 g
5 % glucose solution to	100 ml

higher fatty acids than the emulsifiers previously mentioned. The fatty acids investigated were stearic, oleic, lauric and tridecylic.

The *cottonseed oil emulsions* used in the cat experiments had the composition shown in table 2, except that they contained no fatty acid and that either unsaturated phosphatides (WRETLIND 1957 a) or hydrogenated phosphatides were used. In some experiments we studied emulsions containing triolein instead of cottonseed oil.

The *hydrogenated phosphatides* were prepared as described below. The phosphatide 320 g were dissolved in 600 ml cyclohexane: 3,400 ml absolute alcohol were added, and the precipitated cephalins were filtered off. The filtrate was concentrated under reduced pressure and nitrogen to a volume of 850 ml. To the resulting solution were added 30 mg Raney nickel as catalyst, the solution being then transferred to an autoclave in which it was subjected to hydrogenation at 85–130° C with hydrogen at a pressure of 75–150 atmospheres for two hours. The hydrogenated solution was filtered hot and passed through a column of 500 g Al_2O_3 with a diameter of 45 mm equipped with a heating jacket (75°). The column was eluted with 95 % ethanol. The first fraction of 300 ml was discarded; the next two litres of eluate were added to six litres off acetone and placed in a refrigerator at +4°. The resulting phosphatides were filtered of under reduced pressure, washed with acetone, and dried in a desiccator under reduced pressure. The yield amounted to 30–50 grams. Analysis showed N 1.6–1.7 per cent and P 3.7–3.8 per cent.

Table 2.

Composition of emulsions used for investigating the effects of fatty acids on respiration and blood pressure.

Pluronic F 68 (polypropylene-polyethylene-glucose) obtained from Wyandotte Chemical Corp., Wyandotte, Ill., U.S.A. The emulsions were adjusted to pH 7.3 with 1 N-NaOH.

Fatty acid	1 g
Cottonseed oil	10 g
Phosphatides	1.2 g
Pluronic	0.4 g
5 % glucose solution to	100 ml

Results.

In attempts to determine the LD50 for the carrier emulsions with *cottonseed oil alone*, we found on intravenous injection of a maximal volume – i.e., 60 ml/kg of the 25 % cottonseed oil emulsion with emulsifiers, as shown in table 1 – *into mice* only a transient depression of the respiration rate. It follows that the LD50 is higher than 15 g cottonseed oil per kilogram.

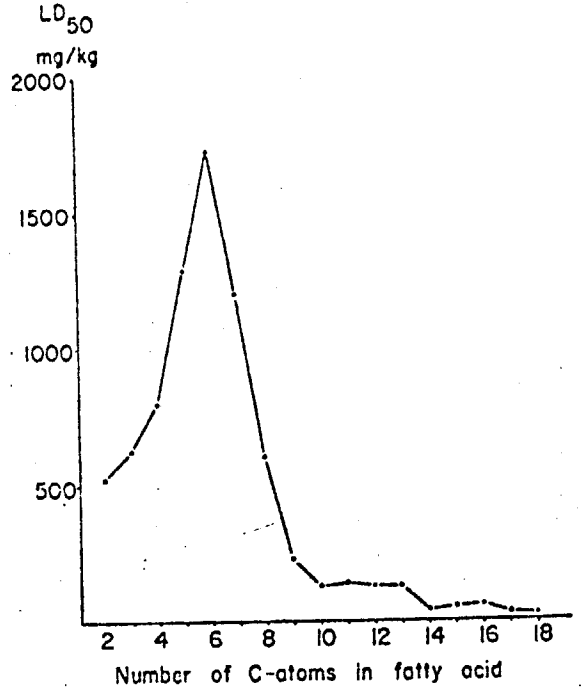

Fig. 1. *Toxicity of saturated fatty acids on intravenous injection into mice.* The abscissa indicates the number of carbon atoms in the fatty acid molecule; the ordinate, LD50 in milligrams per kilogram of body weight. The LD50 values in mg/kg, together with their standard errors, were: acetic acid 525 ± 21; propionic acid 625 ± 33; butyric acid 800 ± 24; n-valeric acid 1290 ± 53; isovaleric acid 1120 ± 30; caproic acid 1725 ± 85; heptylic acid 1200 ± 56; caprylic acid 600 ± 24; nonanoic acid 224 ± 4.6; capric acid 129 ± 5.4; undecylic acid 140 ± 4.2; lauric acid 131 ± 5.7; tridecylic acid 130 ± 7.0; myristic acid 43 ± 2.6; pentadecylic acid 54 ± 3.2; palmitic acid 57 ± 3.4; margaric acid 36 ± 0.3; stearic acid 23 ± 1.3.

Figure 1 shows that the intravenous *toxicities of different fatty acids* differ considerably for mice. Least toxic is caproic acid with six carbon atoms, which has an LD50 of 1,725 ± 85 mg per kg body weight. With an increased or decreased number of carbon atoms in the fatty acid molecule, the toxicity rises. The most toxic of all those investigated was

stearic acid, which had an LD50 of 23 ± 0.7 mg per kg. Approximately the same value was obtained for stearic acid in the rat (21.5 ± 1.8 mg/kg). Oleic acid showed an LD50 of 230 ± 18 mg/kg in mice. This implies that stearic acid is ten times more toxic than the unsaturated oleic acid. The toxicity of norvaleric and isovaleric acids differed slightly, LD50 being 1,290 ± 53 and 1,120 ± 30 mg/kg, respectively.

When the fatty acid solutions and emulsions were injected in amounts near the LD50 dose, the animals immediately had convulsions and collapsed on their sides. Respiration ceased within 1–2 minutes, sometimes after hyperpnoea.

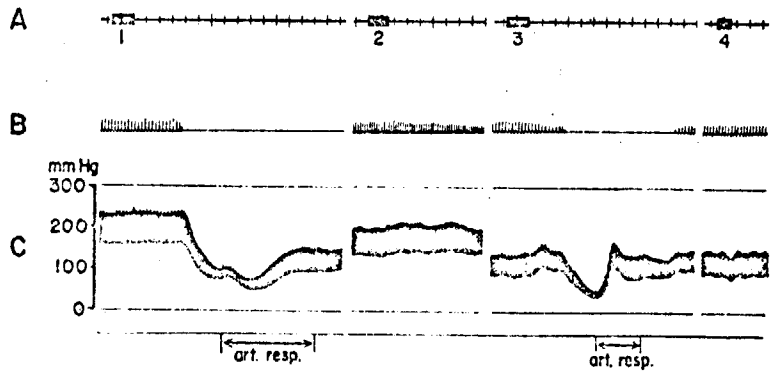

Fig. 2. *Influence of cottonseed oil emulsion on blood pressure and respiration.* Cat, 3.0 kg, under Dial ® anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. "Art, resp." denotes artificial respiration. At 1, 2, 3 and 4, 0.7 ml/kg of 10% cottonseed oil emulsion containing phosphatides was injected intravenously. The interval between 1 and 2 was four minutes, between 2 and 3 four hours and between 3 and 4 two minutes.

Intravenous administration of emulsion with *cottonseed oil or triolein alone to anaesthetized cats* produced various effects, depending on the dose and the emulsifiers. With soy-bean phosphatides and Pluronic F 68 as emulsifiers an initial intravenous injection (0.2–1 ml/kg body weight) sometimes caused a fall of blood pressure as well as apnoea (fig. 2). The latency was usually around 10–40 seconds. Another injection of the same dose invariably had either a less effect or none at all. – The action of these cottonseed oil emulsions is thus characterized by pronounced tachyphylaxis; hence the same or progressively higher doses can be repeated with no demonstrable effects on blood pressure and respiration. – When Pluronic F 68 alone was the emulsifier, 1–3 ml/kg was required to produce an effect with 10% cottonseed oil or triolein emulsion.

Cottonseed oil emulsions produced a rise of pressure in the *pulmonary artery* up to 100 mm Hg (fig. 3). The latency varied from 10 seconds to

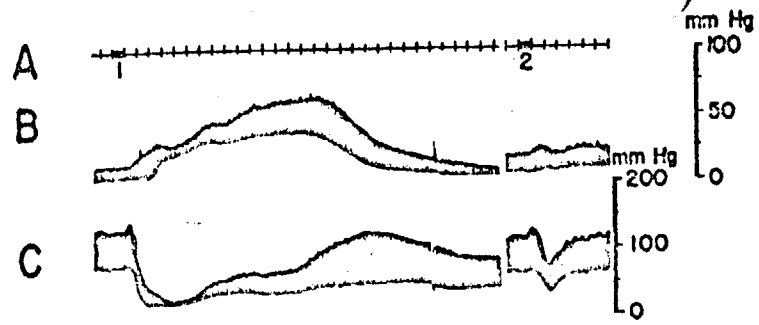

Fig. 3. *Effect of cottonseed oil emulsion on pressure in the pulmonary and carotid arteries.* Cat 3.3 kg, under chloralose anaesthesia with open thorax. A indicates 10-second intervals and injection times; B, the pulmonary artery; C, the carotid artery pressure. At 1 and 2, 0.5 ml/kg of a 10% cottonseed oil emulsion with phosphatides (1.2%) and pluronic (0.2%) as emulsifiers were injected.

2 minutes. Here too the effect declined with repeated injections and ultimately disappeared, although the injected doses were high.

The hypotensive action of these emulsions was substantially less after vagotomy, but in spite of this operation there was an unchanged rise of pressure in the pulmonary artery. This elevation in pressure showed a high degree of tachyphylaxis (fig. 5).

Direct experiments showed also that soyabean phosphatides caused a blood-pressure fall and apnoea, both of which effects exhibited marked tachyphylaxis. With repeated injections, however, the hypotensive action did not completely disappear (fig. 4), a result suggesting that two hypotensive factors were present here.

With cottonseed emulsions containing fatty acid, the first injections were sometimes accompanied by interference with the specific effects of

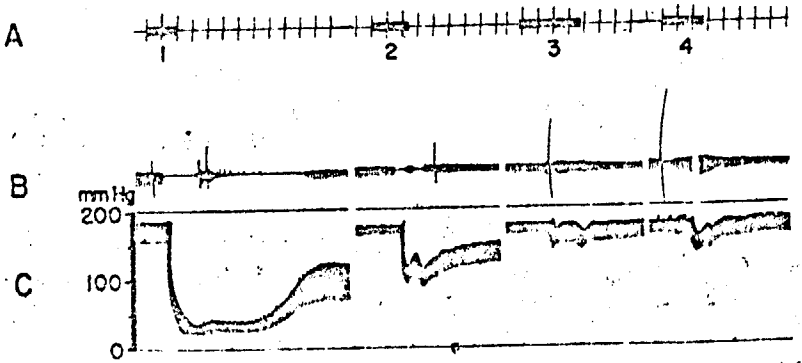

Fig. 4. *Effect of phosphatides on blood pressure and respiration.* Cat, 3.8 kg, under Dial ® anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, the blood pressure in mm Hg. At 1, 2, 3 and 4 4 ml/kg of 2.4% solution of soya bean phosphatides and 0.2% Pluronic F 68 were injected. The phosphatides have a hypotensive action characterized by tachyphylaxis, though a slight effect persists.

the cottonseed oil, but on repeated injection these quickly subsided because of the tachyphylaxis. With fatty acid emulsions containing hydrogenated phosphatides, only the effects of the fatty acids were observed.

Intravenous injection of the *fatty acids* investigated – stearic, oleic, laurfc and tridecylic – had a hypotensive effect on the systemic *circulation*, but raised the pulmonary arterial pressure. The doses required for this action were 5 mg stearic acid, 50 mg oleic acid, 75 mg lauric acid or 60 mg tridecylic acid per kg body weight. All of the fatty acids studied had a negatively inotropic effect on the heart, as shown by the experiments on heart-lung preparations. Similar results were given by oleic acid emulsions

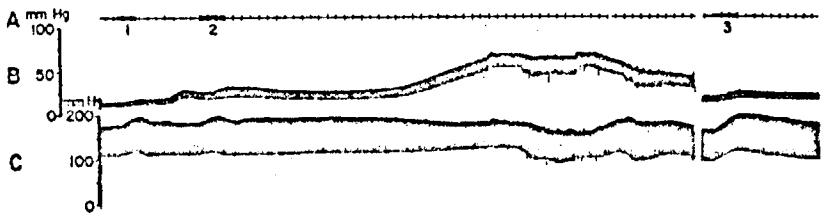

Fig. 5. *Influence of triolein emulsion on blood pressure and pulmonary arterial pressure after vagotomy.* Cat, 4.0 kg, under Dial ⓝ anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, the pressure in the pulmonary artery; C, the arterial blood pressure in mm Hg. Four ml/kg of 10% triolein emulsion with pluronic F 68 only as emulsifier were injected intravenously at 1, and 7 ml/kg at 2 and 3. It will be seen that the emulsion caused a rise of blood pressure in the pulmonary artery with a slight secondary fall in blood pressure, effects that disappeared on repeated injection.

without cottonseed oil as carrier and also by those with unsaturated phosphatides and Pluronic F 68 as emulsifiers. Emulsions with different emulsifiers differed somewhat in their effects. For instance, the effect on pulmonary arterial pressure was far less with hydrogenated than with unsaturated phosphatides. The action on the *respiration* was characterized by hyperpnoea after small doses and apnoea after larger ones. No tachyphylaxis was observed with fatty acid emulsions. In several experiments, indeed, an increased effect was noted on repeated injection of equal doses (figures 6 and 7). – Vagotomy had no effect on falls in blood-pressure produced by the fatty acids studied.

As to the action of *fatty acid emulsions on small intestine from the guinea pig*, our results indicated that the emulsions did not themselves cause any contraction, even in relatively large doses (100 mg/15 ml). They could, however, inhibit the contraction produced by histamine. The degree of inhibition depended on the particular fatty acid contained in the emulsion. Fig. 8 shows the relative potencies of the fatty acids in this respect. It will be seen from the curve that capric and undecylic acids, i.e., those with 10 or 11 carbon atoms, were the most effective. Somewhat more than 15 mg/15 ml of these acids was required for total inhibition of

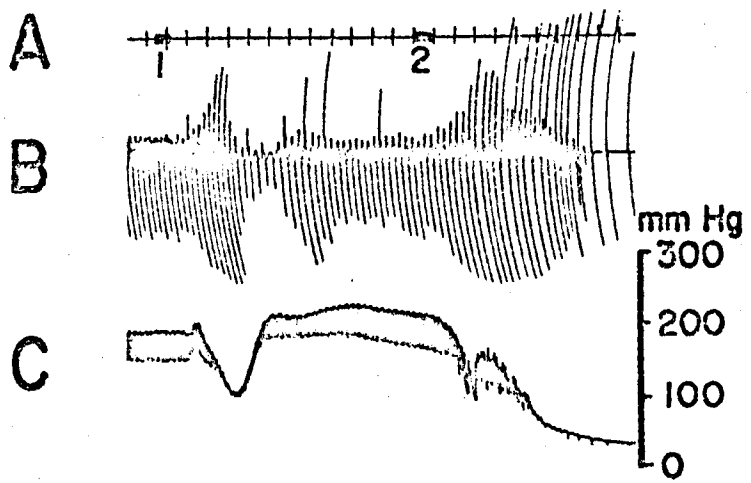

Fig. 6. *Effect of oleic acid emulsion on blood pressure and respiration.* Cat, 4.0 kg, under chloralose anaesthesia with bilateral vagotomy. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. One ml/kg of 10% oleic acid emulsion in cottonseed oil (10%) with soyabean phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at 1 and 2.

the contraction after 0.2 µg histamine. If the fatty acid molecule contained a greater or smaller number of carbon atoms than 10 or 11, the relaxing action on histamine-contracted intestine decreased; for instance, 150 mg of butyric acid was required for total inhibition. Comparison of undecylic acid with its unsaturated homologue, undecylenic acid, revealed no significant difference. The emulsion system alone without fatty acid is inert. No difference was detectable when the lower fatty acids were administered in aqueous solutions instead of in emulsions.

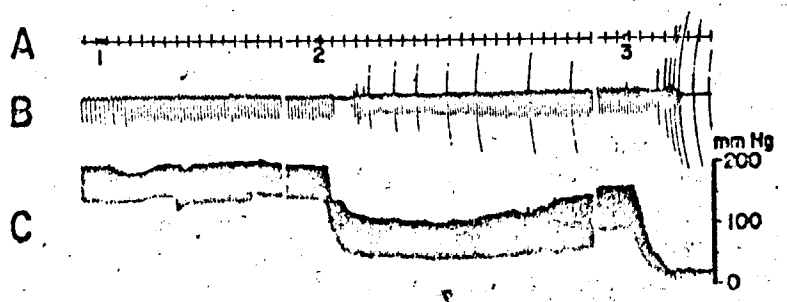

Fig. 7. *Effect of stearic acid emulsion on respiration and blood pressure.* Cat, 4.3 kg, under chloralose anaesthesia. A indicates 10-second intervals and injection times; B, respiration; C, blood pressure in mm Hg. At 1, 2 and 3, a 1% stearic acid emulsion with 10% cottonseed oil as carrier and phosphatides (1.2%) and pluronic F 68 (0.2%) as emulsifiers was injected at a dose of 0.2 ml/kg.

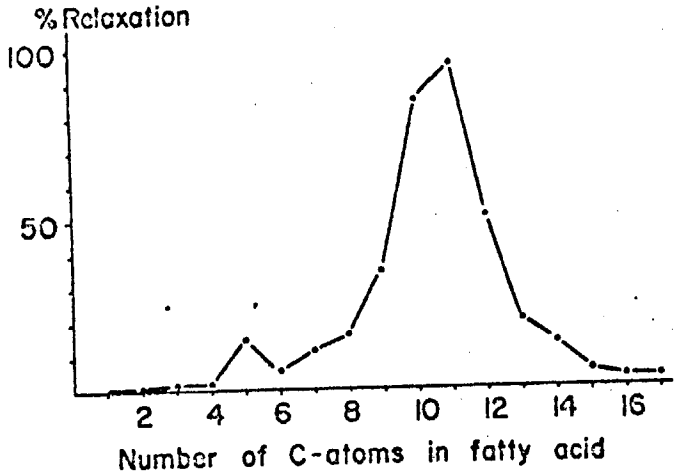

Fig. 8. *Relaxing action of fatty acids on guinea-pig small intestine contracted by histamine.* The fatty acids were administered in the form of emulsion. Abscissa indicates the number of carbon atoms in the fatty acid molecule. Ordinate shows percentage relaxation of guinea-pig intestine contracted by 0.2 µg histamine, after addition of 1.5 mg fatty acid to an organ bath containing 15 ml.

Discussion.

This investigation thus shows that several fatty acids are markedly toxic when administered intravenously. It is mainly the higher fatty acids that show this, (LD50 23 mg/kg) to the greatest extent. With a reduced number of carbon atoms in the molecule the toxicity diminishes, being least for caproic acid with six carbon atoms (LD50 1,724 mg/kg). When compared with the triglycerides of low molecular weight, the free fatty acids thus show the reverse behavior in toxicity; for the LD50 curve for triglycerides reveals a distinct minimum when the fatty acid component has five or six carbon atoms. Thus, hydrolysed fatty acids cannot be responsible for the acute toxic action of the triglycerides.

Our value for the LD50 of acetic acid (525 mg/kg) is closely consistent with that found by WELCH *et al.* (1944) – 380 mg sodium acetate per kg in mice. SAMSON, DAHL & DAHL (1956) described a hypnotic action of fatty acids of low molecular weight (C_2–C_{10}). There seems to be no direct correlation between hypnotic and toxic effects. The ED50 for hypnotic action decreases progressively with an increasing number of carbon atoms in the fatty acid molecule, in contrast to the LD50, the highest dose being that of caproic acid with six carbon atoms and the dose then falling with both increased and reduced numbers of carbon atoms.

The responses of respiration and blood pressure to the fatty acid emulsions differed from those to the triglycerides of low molecular weight

(WRETLIND 1957 a); in cats intravenous injection of more than 5 mg stearic acid or 50 mg oleic acid per kg was followed by apnoea and a fall in blood pressure as well as by convulsions with lethal outcome. Lower doses had a depressor action on blood pressure; the respiration sometimes being stimulated. The dose required for toxic action varied somewhat with the emulsifier employed. The effect on the circulatory system also differed with different emulsifiers. Stearic acid emulsions with phosphatides and Pluronic as emulsifiers raised the pulmonary arterial pressure substantially, and this elevation contributed to the fall of blood pressure in the systemic circulation. With hydrogenated phosphatides there was no demonstrable influence on pulmonary arterial pressure; on the other hand these emulsions had a pronounced effect on heart-lung preparations, so that their hypotensive action may be attributable to direct cardiac depression. Oleic acid emulsions that had been emulsified solely with phosphatides and Pluronic F68, without cottonseed oil, also had a hypotensive and toxic action on heart-lung preparations.

It is accordingly evident that the emulsified higher fatty acids studied had, on intravenous injection, a pronounced toxic action associated with a fall of blood pressure.

PELTIER (1956) determined the toxicity of some neutral fats and free fatty acids in non-emulsified form. He concluded that the toxic effects of fatty acid were caused by damage to the pulmonary capillaries, with extravasation into the alveolar spaces. JEFFERSON (1948) held a similar view. In our experiments on anaesthetized cats we occasionally observed progressive respiratory distress, and blood-tinged froth escaped from the tracheal cannula. At autopsy the lungs were oedematous. – It is worthy of note that the LD50 is more or less the same for non-emulsified oleic acid in rabbits (PELTIER 1956) and emulsified oleic acid in mice (250 mg/kg and 230/kg respectively). SCUDERI (1941) found that 0.33 ml of non-emulsified oleic acid per kg had a toxic action on the dog. The emulsified form had a lethal effect on anaesthetized cats in somewhat smaller doses, i.e., 50–100 mg/kg. However, total amounts substantially greater than 250 mg of emulsified oleic acid per kg can be given by repeatedly injecting sublethal doses.

The fatty acid emulsions had no stimulating action on *small intestine from the guinea pig* – a finding that accorded with results reported by GABR (1956). The emulsions could, however, relax histamine-contracted intestine. This effect was not correlated with their toxic action; rather the reverse, the relaxing effect being maximal for fatty acids with 10 or 11 carbon atoms and decreasing with either a smaller or greater number of carbon atoms in the molecule. Of interest in this connection is the finding of GANLEY *et al.* (1960) that higher fatty acids partially inhibited the

action of serotonin (5-hydroxytryptamine) and the effect in anaphylactic shock in mice.

As to the blood pressure fall that follows injection of emulsions of *cottonseed oil* or *triolein*, it is difficult to determine the mechanism exactly because of the pronounced tachyphylaxis.

If an anaesthetized cat is injected with 6 ml/kg 10% emulsion of cottonseed oil or triolein emulsified with 0.1 or 1.0% Pluronic F 68, a substantial fall in blood pressure as well as apnoea occur after about 10–60 seconds. The cat may even die after this first injection. The pulmonary arterial pressure sometimes rises as high as 100 mm Hg. Our results suggest that the hypotensive effect is elicited mainly via the vagus, for it is practically eliminated by vagotomy (fig. 5).

Since the responses of blood pressure can also be obtained with the soya bean phosphatides alone, it seems probable that the effects observed with the cottonseed oil and triolein emulsions were due to the presence of such phosphatides. That the unsaturated phosphatides were responsible also appears likely, inasmuch as there was no circulatory response when hydrogenated soya bean phosphatides were used as emulsifiers. The fact that cottonseed oil emulsions with Pluronic F 68 as the sole emulsifier produced similar effects on the blood pressure shows that this is not the only explanation. Our observations tend rather to suggest that the effects of intravenously administered fat emulsions are due to a number of factors, such as the properties of the fat, the emulsifiers, the surface tension, size of fat particles and the charge carried by them. On the basis of our present knowledge in this field, it is impossible to say finally which is the principal factor involved.

According to SLEVERS (1958), tachyphylaxis is probably due to specific "receptors" that may take up the active substance, thereby eliciting the pharmacological action. When no more of these receptors are available, the effect ceases. The active substance is subsequently released slowly, and not until the liberation is complete can an effect be once more obtained. In our experiments the recovery period was relatively long, at least four hours. It is not yet possible, however, to say where these receptors are situated or by what mechanism the fat emulsions block them.

Summary.

Pharmacological investigations of the effects of intravenously administered fatty acids of high molecular weight are handicapped by the fact that such acids are insoluble in water at neutral reaction. By utilizing fat particles in an emulsion as carriers of these fatty acids, it has been possible

in our investigation to study the toxicity and certain pharmacological effects of such acids when given intravenously. Although emulsions of the carrier fat are devoid of toxicity, they exhibit peculiar pharmacological effects characterized by pronounced tachyphylaxis. In this investigation the following observations were made.

The LD50 by intravenous injection into mice was determined for saturated *fatty acids* from C_2 to C_{18}. Of these, stearic acid emulsion showed the highest toxicity: LD50 23 mg/kg body weight. With a decreasing number of carbon atoms in the fatty acid molecule the toxicity fell, reaching a minimum for caproic acid: LD50 1,725 mg/kg. As the number of carbon atoms declined beyond that point, so did the toxicity rise again, the LD50 amounting to 525 mg/kg for acetic acid.

The LD50 for stearic acid was ten times less than for its unsaturated analogue, oleic acid. For the *cottonseed oil emulsion* employed as "carrier" the LD50 was indeterminable, being higher than 15 g cottonseed oil per kilogram.

The pharmacological effects of emulsions of *cottonseed oil and triolein* on respiration and blood pressure were investigated by intravenous injection into cats. With soya bean phosphatides and Pluronic F 68 as emulsifiers a blood pressure fall and apnoea as well as an elevation of the pulmonary arterial pressure were observed. These effects were also noted for fat emulsions emulsified solely with Pluronic F 68 and for unsaturated soya bean phosphatides alone. Emulsions with hydrogenated phosphatides, on the contrary, did not produce such effects. The action on the circulation and respiration showed marked tachyphylaxis.

The effects of higher *fatty acid emulsions on circulation and respiration* were characterized by a fall in blood pressure as well as hyperpnoea or apnoea. Such emulsions also had a negatively inotropic effect on the heart.

Fatty acid emulsions had no direct action on *small intestine* from the guinea pig, but inhibited contractions elicited by histamine.

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OBSERVATIONS ON SOME EFFECTS OF THE SODIUM SALTS OF CERTAIN MONOCARBOXYLIC ACIDS ON ESTABLISHED CELL LINES[1]

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There appears to be very little information in the literature pertaining to concentrations of various air pollutants or tobacco-smoke constituents that may be toxic or nontoxic to tissue cells cultivated in vitro. We have undertaken the task of ascertaining these levels and this report records observations concerning the first few of many known pollutants.

The effects of the sodium salts of 10 monocarboxylic acids (formate, acetate, propionate, butyrate, valerate, caproate, oenanthylate, caprylate, caprate, and benzoate) on several established cell lines (HeLa, strain L, human lung, human skin) were studied.

All of these compounds at a concentration of 10 mg % were toxic to the cell lines tested with the exceptions of formate, acetate, and benzoate on strain L, and of valerate and caprylate on human lung.

All the compounds either stimulated proliferation or had no significant effect at 1 mg % except caproate and benzoate, which were toxic to human lung and human skin cells, respectively.

Introduction

It has been recognized for some time that polluted air and tobacco smoke contain acidic components. Most earlier investigations on the occurrence of acids such as acetic, formic, and butyric (1), were based on qualitative determinations and only recently have quantitative studies been made. Buyske and colleagues (2) undertook a quantitative identification of the individual steam-volatile monocarboxylic acids composing 5–8% of the particulate phase of tobacco smoke. The following experiments are concerned with these acids.

Although there is little evidence of carcinogenic activity by any of these compounds (3, 4), they may be of biological and medical importance otherwise. Some of them, especially those with a carbon chain length of C_8 to C_{14}, exhibit rather pronounced antitumor activity (5–7).

Ciliastatic action of tobacco smoke has been reported by numerous investigators (8–13). According to Wynder *et al.* (13), the effect seems to be due to some extent to the acidic and phenolic fractions of smoke condensate. They found that the ciliatoxicity produced by formic acid, acetic acid, propionic acid, benzoic acid, and formaldehyde was more pronounced, and that produced by acetaldehyde, benzaldehyde, *n*-butyric acid, and oxalic acid was less pronounced, than the effects of phenol.

Some of the steam-volatile organic acids were chosen for these studies to obtain information regarding their toxicity in cells cultivated in vitro. This additional information concerning some of the biological effects of these constituents of polluted air and tobacco smoke should prove to be of interest and value to other investigators.

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Materials and Methods

Human skin cells (NCTC Clone 2414), strain L cells (NCTC Clone 929), and HeLa cells were used in testing some of the compounds, and human fetal lung cells were utilized in all the tests. The cells were cultured in NCTC 109 medium supplemented with 10% horse serum (14–16) to which was added, at desired concentrations, the compound under investigation.

Short-term experiments, extending to 19 days, were conducted to ascertain toxic and nontoxic concentrations.

Cultures were replicated in T-15 flasks by the usual procedures (17). Two milliliters of cell suspension were added to each flask. After an adjustment period of 48 hours the flasks were divided into control and experimental groups. Thereafter, the medium was changed every 48 hours and the cells in each of three flasks, selected at random from each group, were counted. Counts were made with a Coulter electronic counter.

Erythrosin B was used for testing viability (18). Microscopic examinations were made to ascertain morphological changes. In some instances photographs were taken to record any alterations observed.

Results and Discussion

The results of the experiments are presented in Table I. Some explanation is necessary concerning the two terms "effect" and "morphological changes".

Several terms were used to describe the effects of a compound upon the cells. The effect was described as "toxic" if the cells died or if there was a decrease in their population. If proliferation occurred, but to less extent than that of the control cells, the term "retarding" was used. An effect resulting in a significantly greater population than that of the controls was considered "stimulating". "Negligible" denoted neither significant inhibitory nor stimulatory effects. The table does not indicate, however, the occasional instances when during the first 48 to 96 hours the compound appeared to stimulate growth, although often this period of stimulation was followed by a retarding effect.

Morphological alterations, observed under ordinary microscopic examination, were varied. One of the most prominent and frequent changes was a tendency of the cells to take on a spherical shape, and this was usually followed by detachment. An increase in number and size of vacuoles as well as in granule formation was not uncommon. Experimental cells sometimes appeared to be enlarged, and were frequently filamentous and spindle-shaped (at least, more so than control cells). Giant cells developed occasionally and in some instances blebs appeared on the surface. In the table, the terms used for morphological changes indicate only that changes were or were not observed, and are in no way descriptive. When the appearance was recognized as definitely altered, it is indicated in the table that the alteration took place within a certain

time or time range. If no change could be detected, the term "none" is used, although this is based only on observations made at a magnification of about 400 times. Fixation of the cells, which occurred at times when they were exposed to the higher concentrations of some compounds, was often difficult to recognize, since appearance was not appreciably altered and detachment did not necessarily occur. However, viability or indications of fixation could be ascertained by means of erythrosin B.

Observations were often made at 24-hour intervals and routinely made at least every 48 hours.

A concentration of 2000 mg % was used only with sodium formate and acetate. Sodium formate, at this concentration, appeared to be more toxic to strain L cells, which were dead within 24 hours, than to human skin cells, which continued to live for approximately 96 hours. Both types of cells displayed obvious changes after the first 24 hours. Sodium acetate at a concentration of 2000 mg % produced a declining population in human skin cells, but in strain L cells a retarding effect occurred, in comparison with controls. Again, both cell types showed morphological changes within 24 hours.

The results obtained when cells in vitro were subjected to a range of concentrations of the salts are presented in Table I. In concentrations of 1000 mg %, acetate resulted in either a retarded rate of proliferation or a declining population. Formate retarded proliferation of strain L cells and caused a progressively declining population of human skin cells.

It is interesting to note that all concentrations (10 mg % and above) of *formate* and *acetate* had a growth-retarding effect, except on strain L cells, and that the effect on HeLa cells appeared to be negligible. (The additional Na content at this and lower concentrations of the salts is negligible compared with the total Na content of the medium, e.g. the Na content in 10 mg % Na formate is about 5 mg %. The total Na content of the medium plus 10% serum was about 400 mg %.) Proliferation in strain L seemed to be enhanced when the cells were exposed to this concentration. Formate at 1 mg % also appeared to stimulate proliferation in human skin cells. Usually, if the so-called "retarding" effect was comparatively slight no morphological changes were observed.

Sodium *propionate* appeared to be toxic to human lung cells only in concentrations above 10 mg %. No other cell lines were tested.

Sodium *butyrate* in concentrations up to 1 mg % seemed to have very little if any effect on the three lines indicated. At 10 mg % and above it became quite toxic.

Sodium *valerate* at 10 mg % was toxic to all cell lines except human lung cells, on which there was very little effect, if any.

Only human lung cells were exposed to *caproate*, which was toxic, especially above 10 mg %.

The results indicate that *oenanthylate* at a concentration of 1 mg % had no effect on human lung cells but had a retarding effect and was quite toxic above 10 mg %.

TABLE I

The effects of various concentrations of the sodium salts of some monocarboxylic acids on several established cell lines (explanation of "Effect" and "Morphological changes" given in the text)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure (× 10⁶)	Time to maximum population (hours after exposure)	Maximum population (× 10⁶)	Effect	Morphological changes
Sodium formate	Human skin (NCTC 2414)	0	0.152	144	0.411	—	—
		1	0.152	144	0.492	Stimulating (?)	None
		10	0.152	96	0.309	Retarding	None
		100	0.152	144	0.271	Retarding	Within 48 hours
		1000	0.152	—	—	Dead within 144 hours	Within 24 hours
		2000	0.132	—	—	—	—
	Strain L (NCTC 929)	0	0.132	144	1.084	—	—
		1	0.132	144	1.174	None	None
		10	0.132	144	1.640	Stimulating	None
		100	0.132	144	0.903	Retarding (?)	None
		1000	0.132	144	0.140	Retarding	Within 48 hours
		2000	0.132	—	—	—	—
	Human lung	0	0.279	192	0.872	—	—
		1	0.279	144	0.851	None	None
		10	0.279	144	0.635	Retarding	Within 144 hours
		100	0.279	144	0.509	Retarding	Within 96–120 hours
		1000	0.279	—	—	Dead within 48 hours	Within 24 hours
		2000	0.356	240	0.760	—	—
	HeLa	0	0.356	144	0.792	—	—
		1	0.356	144	0.702	Negligible	None
		10	0.356	168	0.668	Retarding (?)	None
		100	0.356	240	—	Retarding	Within 240 hours
		1000	0.356	—	—	Dead within 24 hours	Within 24 hours
		2000	0.578	216	2.10	—	—
Sodium acetate	Human skin (NCTC 2414)	0	0.578	216	1.95	—	—
		1	0.578	216	1.82	None	None
		10	0.578	144	0.351	Retarding	None
		100	0.578	—	—	Retarding	None
		1000	0.578	—	—	Toxic	Slight
		2000	0.132	144	1.084	—	—
	Strain L (NCTC 929)	0	0.132	144	1.087	—	—
		1	0.132	144	1.379	None	None
		10	0.132	144	0.666	Stimulating	None
		100	0.132	120	0.254	Retarding	None
		1000	0.132	96	—	Retarding	Negligible

TABLE I (*Continued*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
	Human lung	0	0.284	192	0.830	—	—
		1	0.284	216	0.792	None	None
		10	0.284	192	0.710	Retarding	None
		100	0.284	96	0.531	Retarding	Within 48 hours
		1000	0.284	—	—	Toxic	Within 12–24 hours
	HeLa	0	0.142	192	0.516	—	—
		1	0.142	192	0.510	None	None
		10	0.142	192	0.485	Retarding	Slight
		100	0.142	144	0.458	Retarding	Slight
		1000	0.142	192	0.292	Retarding	Within 144 hours
Sodium propionate	Human lung	0	0.310	144	1.060	—	—
		1	0.310	196	1.318	Stimulating	None
		10	0.310	196	1.149	None	None
		100	0.310	144	0.399	Retarding	Within 96 hours
		1000	0.310	—	—	Dead within 48 hours	Within 24 hours
Sodium butyrate	Human skin (NCTC 2414)	0	0.578	216	2.10	—	—
		1	0.578	192	1.952	None	None
		10	0.578	192	1.222	Dead within 144 hours	Within 48 hours
		100	0.578	—	—	Dead within 48 hours	Within 48 hours
		1000	0.578	—	—	Dead (24–48 hours)	Within 24 hours
	Strain L (NCTC 929)	0	0.132	144	1.380	—	—
		1	0.132	144	1.290	None	None
		10	0.132	48	0.490	Retarding	Within 48 hours
		100	0.132	—	—	Dead within 48 hours	Within 24–48 hours
		1000	0.132	—	—	Dead (24–48 hours)	Within 24 hours
	Human lung	0	0.272	144	0.840	—	—
		1	0.272	144	0.793	None	None
		10	0.272	96	0.462	Retarding	Within 96–120 hours
		100	0.272	—	—	Dead within 48 hours	Within 24–48 hours
		1000	0.272	—	—	Dead within 48 hours	Within 24 hours

TABLE I (*Continued*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure ($\times 10^6$)	Time to maximum population (hours after exposure)	Maximum population ($\times 10^6$)	Effect	Morphological changes
Sodium valerate	Human skin (NCTC 2414)	0	0.172	144	0.714	—	—
		1	0.172	192	0.682	None	None
		10	0.172	192	0.324	Retarding	Within 24 hours
		100	0.172	—	—	Dead within 96 hours	Within 24 hours
		1000	0.172	—	—	Dead within 24 hours	Within 24 hours
	Strain L (NCTC 929)	0	0.126	144	1.380	—	—
		1	0.126	144	1.300	None	None
		10	0.126	144	0.632	Retarding	Within 48 hours
		100	0.126	144	0.300	Retarding	Within 24–48 hours
		1000	0.126	—	—	Dead within 48 hours	Within 24 hours
	Human lung	0	0.281	192	1.104	—	—
		1	0.281	192	1.150	None	None
		10	0.281	192	1.090	None	Within 96 hours
		100	0.281	—	—	Toxic	Within 72 hours
		500	0.281	—	—	Toxic	Within 24 hours
Sodium caproate (hexanoic Na salt)	Human lung	0	0.344	144	0.990	—	—
		1	0.344	168	0.682	Retarding	Within 120 hours
		10	0.344	168	0.515	Retarding	Within 120 hours
		100	0.344	—	—	Dead within 96 hours	Within 24 hours
		500	0.344	—	—	Dead within 96 hours	Within 24 hours
Sodium oenanthylate (heptanoic Na salt)	Human lung	0	0.225	96	0.491	—	—
		1	0.225	96	0.480	None	None
		10	0.225	96	0.346	Retarding	Within 96 hours
		100	0.225	—	—	Dead within 48 hours	Within 24–48 hours
		500	0.225	—	—	Dead within 24 hours	Within 4 hours
Sodium caprylate (octanoic Na salt)	Human lung	0	0.310	144	0.660	—	—
		1	0.310	144	0.737	Stimulating (?)	None
		10	0.310	96	0.451	Retarding	Within 96–120 ho
		100	0.310	—	—	Dead (48–96 hours)	Within 24 hours
		500	0.310	—	—	Dead (24–48 hours)	Within 24 hours

TABLE I (*Concluded*)

Substance tested	Cell line	Concentration of substance tested (mg %)	No. of cells/ml at exposure (× 10⁴)	Time to maximum population (hours after exposure)	Maximum population (× 10⁴)	Effect	Morphological changes
Sodium caprate (decanoic Na salt)	Human skin (NCTC 2414)	0	0.156	216	0.710	—	—
		1	0.156	216	0.955	Stimulating	None
		10	0.156	144	0.233	Retarding	Within 96 hours
		100	0.156	—	—	Dead within 48 hours	Within 24 hours
		1000	0.156	—	—	Dead (24–48 hours)	Within 24 hours
	Strain L (NCTC 929)	0	0.175	144	1.255	—	—
		1	0.175	144	1.182	None	None
		10	0.175	120	0.355	Retarding	Within 48 hours
		100	0.175	—	—	Dead within 48 hours	Within 24 hours
		1000	0.175	—	—	Dead within 24 hours	—
	Human lung	0	0.424	144	1.110	—	—
		1	0.424	144	0.976	None	None
		10	0.424	120	0.276	Retarding	Within 72 hours
		50	0.424	—	—	Dead within 96 hours	Within 24–48 hours
		100	0.424	—	—	Dead (48–72 hours)	Within 24 hours
Sodium benzoate	Human skin (NCTC 2414)	0	0.156	216	0.710	—	—
		1	0.156	216	0.650	Retarding (?)	None
		10	0.156	144	0.517	Retarding	None
		100	0.156	144	0.434	Retarding	Within 120 hours
		1000	0.156	—	—	Dead (24–48 hours)	Within 12–24 hours
	Strain L (NCTC 929)	0	0.175	144	1.255	—	—
		1	0.175	144	1.306	None	None
		10	0.175	144	1.150	None	None
		100	0.175	120	0.805	Retarding	Within 96 hours
		1000	0.175	—	—	Dead (48–96 hours)	Within 12–24 hours
	Human lung	0	0.189	168	1.146	—	—
		1	0.189	168	1.070	None	None
		10	0.189	144	0.474	Retarding	Within 48–72 hours
		100	0.189	120	0.373	Retarding	Within 48–72 hours
		1000	0.189	—	—	Dead within 72 hours	Within 24 hours

Caprylate at 1 mg % actually enhanced proliferation of human lung cells but was toxic at 10 mg % and above.

Sodium *caprate* stimulated proliferation of human lung cells but appeared to have no effect in concentrations of 1 mg %. Greater concentrations were toxic in all cases.

Sodium *benzoate* at 1 mg % appeared to have very little effect on any of the three lines tested, but except for strain L it was toxic above this concentration.

Aromatic polycyclic hydrocarbons, some of which are carcinogenic, may be adsorbed by soot particles in polluted air (19–22). These adsorbed carcinogenic hydrocarbons are considered biologically inactive (22, 23). Organic acids such as those occurring in tobacco smoke and polluted air may act as solvents for these carcinogenic hydrocarbons. Therefore, if soot and tobacco smoke should occur simultaneously in the lungs, the carcinogens possibly might be eluted from the soot by the organic acids of the smoke. The eluted carcinogens might then become biologically active (22).

Smog and tobacco smoke are known to be irritating to the mucous epithelium of the respiratory tract. This irritant nature may be due in part to acids and could facilitate further action, especially on the basal cells, by carcinogens.

Our interest in toxicity levels for the numerous air pollutants and tobacco-smoke constituents is based on our intention to investigate further their actual effects on basic mechanisms within cells. Additional information concerning biological effects of these constituents should prove to be of interest and value to other investigators.

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SOME RESULTS OF RESEARCH ON ANTISEPTICS IN FOOD PRODUCTS FROM THE MICROBIOLOGICAL VIEWPOINT*

By L. Paix[1] and J. H. H. Mol[2]

For a long time, the attention of hygienists has been attracted to the study of the dangers which may result from the absorption of antiseptics increasingly being used in food (2), (3), (4).

However, their number, their variety and their efficacy continuously increasing makes the task of the chemist difficult: how is he is to find, then identify the presence of substances whose concentration is of the order of milligrams per kilogram or liter?

The publications of Doctor Mossel on the aspecific detection of antiseptics by microbiological means opens up a new method, full of promise for the study of this problem.

The method used consists of innoculating into appropriately prepared substratum, a suspension of Saccharomyces cerevisiae in the form of a simple baking leaven diluted in a certain way. This is transferred into Einhorn tubes which are kept at 24°C. After 24, 30, 48 or 67 hours according to the case, the volume of the released gas is measured (1).

If the substance in question contains an antiseptic in an efficacious concentration, fermation will be stopped or even nonexistant.

Different food-products were used in the course of this work: beer, jam, milk, lemonade, margarine, meat and ice cream.

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*Work done under the direction of Doctor D. A. A. Mossel in the Laboratory of the "Central Institut vooz Voedingsonderzoek T.N.O." in Utrecht, Netherlands (Director Dr. M. van Eekelen).

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Two general remarks:

The sensitivity of the test and the concentration of the innoculative substance vary inversely: this follows from combinations interferring between the protein and antiseptic substances and diminishing the efficacy of the latter.

Furthermore, in the cases of product naturally rich in germs, one must counteract the microbial antagonism by using more concentrated (10^4 cells/ml in the innoculated solution) suspensions of S. cerevisiae.

These two points of views must obviously be reconciled. It should also be noted that a concentrated innoculation allows for readings to be made more rapidly.

It is absolutely necessary, no matter what the experimental substance is, to do a blank run, parallel to the experiment, whether this is an identical substrate of certain origin or an artificial solution.

The fermentation capacity of the leaven must be verified under the exact conditions of the experiment: in this way, one can be certain that the source used is active, that the substrate contains sufficient growing factors and no natural inhibitors and that the danger of positive mistakes is avoided.

1. Beer (5)

Beer, being generally a very poor medium for allowing active fermentation, 5 ml of a sterile solution of glucose (50 g + 100 ml H_2O) and 10 ml of Wickerham's nutritive solution (mineral salts, vitamins) is added per 100 ml of beer. This is sterilized by filtration and stored in the refrigerator.

Method of Operation

The experiments were done with the following: a Dutch beer (known to be without antiseptic agents as it is; the same beer to which was added

$HgCl_2$ in amounts of 5, 10, 20 mg Hg/liter; the same beer to which was added a phemylmercuric derivative in amounts of 0.5 and 1.0 mgr Hg/liter; the same beer to which was added ethyl monobromacetate in amounts of 0.5 mg/liter; the same beer to which actidione was added in a concentration of 0.2 mg/liter; a light Belgian beer as is; a special Belgian beer as is.

50 ml of each sample of decarbonated beer as is or to which was added antiseptic, were fermented under sterile conditions with 2.5 ml of a glucose solution (50 g + 100 ml H_2O) and 5 ml of Wickerham solution.

The substance to be innoculated is prepared in the form of a suspension of 1 g of baking leaven (in solid form) (aged for 3 days and kept on ice) diluted in 9 ml of sterile physiologic water (0.85% NaCl) (this solution contains an average of 10^9 cells/ml): this is successively diluted in sterile physiologic water until a solution of 10^4 cells/ml is obtained. From this last solution 0.5 ml is taken to be added to each sample.

The Einhorn tubes are filled and placed in an incubator at 24°. Readings of the gaseous volume were taken at 48, 67, and 72 hours.

There were two Einhorn tubes for each attempt.

The gaseous volumes indicated in the table were calculated by multiplying the height of the released gaseous volume by the approximate value of 1.5.

Conclusions

All the samples to which antiseptic or antifungus agents agents gave positive results. Using the blank run as a point of departure, we arbitrarily considered an inhibition of fermentation (measured as gaseous volume) greater than or equal to 50% as a positive result.

RESULTS

	Milieu	Gaseous volume in cm^3 after: 48 h.	67 h.	72 h.
1	Dutch beer	0	17,5	
2	+ glucose + W.D.	0	17,5	
3	Dutch beer	0	0	1,5
4	+ glucose + W.D. + 10 mg Hg/liter ($HgCl_2$)	0	0	1,5
5	Dutch beer	0	0	0
6	+ glucose + W.D. + 10 mg Hg/liter ($HgCl_2$)	0	0	0
7	Dutch beer	0	0	0
8	+ glucose + W.D. + 20 mg Hg/liter ($HgCl_2$)	0	0	0
9	Dutch beer	0	0	0
10	+ glucose + W.D. + 0.5 mg Hg/liter (phenylmercuric salt)	0	0	0
11	Dutch beer	0	0	0
12	+ glucose + W.D. + 1 mg Hg/liter (phenylmercuric salt)	0	0	0
13	Dutch beer	0	0	0
14	+ glucose + W.D. + 0.5 mg/liter ethyl monobromacetate	0	0	0
15	Dutch beer	0	0	0
16	+ glucose + W.D. + 0.2 mg/liter actidione	0	0	0
17	Light Belgian beer	5	17,5	
18	+ glucose + W.D.	9	17,5	
19	Special Belgian beer	0	0	0
20	+ glucose + W.D.	0	0	0

The special Belgian beer clearly appeared to contain a fermentation inhibitor. Further, this was found through chemical means to be Hg in an amount of 1.7 mg/liter.

An amount of Hg of 5 mg/liter introduced in the form of the $HgCl_2$ seemed to be a concentration approaching the threshold of perceptibility, while an amount of 0.5 mg/liter of Hg introduced in the form of phenylmercuric salt gave a positive result. This demonstrates well that the antiseptic power of Hg depends on the form of the compounds in which it is introduced, and it is superior to the microbiological method in its ability to measure the actual effectiveness of an antiseptic and to do this in a milieu to which the antiseptic has been added.

2. Jams

The osmotic pressure found in jam was found to be too high for the acitivity of S. cerevisiae: this is why a dilution of 1 to 5 was judged necessary.

The difference is made up in the diluted jam with leavening agents using a dilution of 1:5 with a sterile solution of 0.30% leavening extract.

Certain jams, principally those with citrus fruit bases, contain terpene peroxides, natural inhibitors of S. cerevisiae. In order to avoid positive errors, before sterilization, we put into the Einhorn tubes 1 to 1.5 g of liver cut into small pieces; the variation of the potential redox which resulted from this enabled us to get around this problem. However, it should be noted that the sensitivity of this method is slightly diminished by the introduction of organic matter.

The samples were adjusted to pH 5.5±0.1 in order to inactivate the benzoic and sulfuric acids legally used in the Netherlands.

Method of Operation

The experiment was done using the following: a Dutch marmelade as is; the same marmelade with liver added; the same marmelade to which liver and ethyl monobromacetate in amounts of 1mg/kg were added; the same marmelade to which liver and ethyl monobromacetate in amounts of 2 mg/kg were added.

Each sample of 50 g of jam as is or to which was added ethyl monobrom-acetate was diluted with 200 ml of 0.30% solution of leavening extract (sterile). These were mixed in the "Turmix" for 5 minutes, pH was adjusted to around 3.5, pasteurized for 1 hour at 80°C (by immersion in water bath of 85°C, with as a control, a container of the same sort containing the same volume of distilled water and into which a thermometer was plonged).

Then the pH was adjusted to 5.5, aseptically with the aid of 10% NaOH. Into every 50 ml of each sample, we innoculated 0.5 ml of a suspension of the leaven containing about 10^4 cells/ml, filling the Einhorn tubes (containing liver or not according to the sample), and put them in the incubator at 24°C.

Readings were made after 24, 30, and 36 hours.

RESULTS

	Gaseous volume in cm^3 after:	
	24 h.	30 h.
21 Water	1.5	17.5
+ glucose 22 + leavening extract	1	13
23 Jam	1,5	16,5
as is 24 Dilution 1: 5	1,5	17.5
25 Jam	15	17.5
as is 26 Dilution 1: 5 Liver in Einhorn tubes	13.5	17.5

Results Con't

		24 h.	30 h.
27	Jam + 1 mg/kg ethyl monobromacetate	2.5	17.5
28	Dilution 1:5 Liver in Einhorn tubes	2.5	17.5
29	Jam + 2mg/1g ethyl monobromacetate	0	5.5
30	Dilution 1:5 Liver in Einhorn	0	8.5

CONCLUSIONS

In the sample containing 2mg of ethyl monobromacetate per kilogram, fermentation is clearly stopped. In the sample with 1mg/kg, no conclusion can be made, fermentation occurring in the same way as in the blank run and in the sample of jam as is without liver added to the "Einhorn" tube.

The presence of the liver would give clear proof of its effectiveness if the attempt without the liver hadn't fermented in the same way as the blank run.

Nonetheless, sight must not be lost of the fact that the blank run, containing an artificial solution, didn't contain as many expanding factors as a jam rich in fruit, and we may wonder if the effects of these factors didn't effectively counterbalance those of the natural inhibitors.

In this particular care, the most favorable time for reading was at 30 hours of incubation.

From the point of view of sensitivity, we can note that in the case of jam, the sample containing 2mg of the monobromacetate ester per kilogram gave a clear positive result, while in the case previously discussed (beer), an even more clear positive result with only 0.5mg of this ester per liter. This result is predictable and normal because in beer, the method was put into effect in a way so as to push as far as possible the threshold of perceptibility: first of all, by introducing (the milieu is ready for it) a less concentrated suspension of the leavening agent (10^4 cells/ml), and also by introducing supplementary expanding factors in the form of Wickerham's solution. This

gives to the leavening extract, besides the advantage of being of a definite composition, that of avoiding having to add to the bear (naturally poor in proteins and amino acids with thiol functions) sulf-hydride compounds which neutralize the effect of certain antiseptics and even lower the sensitivity of the test.

3-Milk (6)

Milk, a true breeding ground for microorganisms cannot be sterilized by heat before the microbiological assay; some antiseptics like the monobromacetic ester and chloropicrine are not resistant to such a treatment.

As pointed out above, in order to combat the microbial antagonism, a massive implantation of 10^6 cells/ml must be used in the experiments.

The necessary hydrocarbonated supplement in the case of milk is formed by glucose from a sterile solution (50g + 100ml H_2O) from which 5ml per 100ml of milk is added.

The use of S. cerevisiae presents two advantages here:

a) Antibiotics used in veterinary therapy have no effect on it.

b) The reductions of pH (to 4.5 at the most) frequently occurring in summer milk are not harmful to its development; a stopper is thus not necessary.

Method of Operation

The experiments were done using: pasteurized milk as is; pasteurized milk + 1mg ethyl monobromacetate per liter; pasteurized milk + 2mg ethyl monobromacetate per liter; 50ml of each sample of milk as is or to which antiseptic agents were added were enriched under sterile conditions with 2.5ml of a glucose solution (50g + 100ml H_2O).

These are inplanted with 0.5 ml of a suspension of 10^6 cells of leaven per ml, put into Einhorn tubes and placed in an incubator at 24°C.

Readings are made after 24 and 48 hours.

RESULTS

		Gaseous volume in cm^3 after:		
		24 h.	40 h.	48 h.
31	Pasteurized milk	coag.	17.5	
32	+ glucose	coag.	13.5	17.5
33	Pasteurized milk	0	0	2.5
	+ glucose	0	0	2.5
34	+ 1 mg/liter ethyl monobromacetate			
35	Pasteurized milk	0	0	0
	+ glucose			
36	+ 2 mg/liter ethyl monobromacetate	0	0	1

Conclusions:

The sample to which ethyl monobromacetate was added showed no fermentation.

4 - Lemonade (7)

The remark made about jam concerning the natural inhibitors of terpene peroxides is equally applicable in this case.

Certain drinks sold under the designation "lemonade" are purely synthetic products of sugar bases, citric acid and artificial coloring and aroma; the growth factors are obviously absent and the addition of leaven proves to be indispensable.

It may be wondered to what degree the colorings used in this kind of drink innibit the development of S. cerevisiae.

The case of eosin was studied; tolerable quantites of bromine were introduced in this form up to 50 mg Br/liter which represents 50 times the detectable quantity of antiseptic bromine.

In the Netherlands, sulfuric and benzoic acids being permitted in lemonade, the pH is fixed at 5.5 $\pm$ 0.1 in order to inactivate them.

Method of Operation.

The experiment were done using:

a Dutch lemonade with a cherry base, as is;

the same lemonade to which was added 0.2 mg ethyl monobromacetate per liter;

the same limonade to which was added 0.4 mg ethyl monobromacetate per liter.

To 50 ml of each of the lemonade samples as is, or to which antiseptec was added, we added 5 ml of a sterile solution of leaven extract (25 gr + 100 ml H_2O) which brought the pH aseptically to 5.5.

A blank run was done at the same time on water (pH 5.1) to which 1 ml of the leaven extract (25 g + 100 ml H_2O) and 5 ml of glucose (50 gr + 100 ml H_2O) per 100 ml of water was added.

Each amount of 50 ml was implanted with 0.5 ml of a suspension of leavening agent of 10^6 cells/ml. The Einhorn tubes were filled and kept at 24°C.

Readings were made after 24, 30 and 48 hours.

Results

		Gaseous volume in cm^3 after:	
		24 h.	30 h.
37	Lemonade	15	17.5
38	+ leaven extract pH 5.4	11.5	17.5
39	Water	1.5	7
40	+ leaven extract + glucose pH 5.1	3	5.5
41	Lemonade	1.5	4.5
42	+ leaven extract + 0.2mg/liter ethyl monobromacetate pH 5.4	1	4
43	Lemonade	0	1.5
44	+ leaven extract + 0.4mg/liter ethyl monobromacetate	0	2.5

CONCLUSIONS:

The most favorable reading was made after 30 hours of incubation.

The blank run did not ferment as quickly or as strongly as the plain lemonade, but we must no lose sight of the richness of fruits in growth factors; here, we are concerned with a lemonade having a fruit juice base.

The concentrations of bromine derivatives additions are clearly inhibitory.

5-Margarine

The research being done on "non-fatty" material, the liposoluble colorings used interferred in no way.

Method of Operation

The experiments were done using: the non-fats of a diluted margarine 1:10; the same non-fats to which was added 2mg/liter of ethyl monobrom-acetate, then diluted to 1:10.

A sufficient quantity of margarine is introduced into a sterile receptacle and melted at 43°C; the serum is taken off aseptically.

The samples of serum as is or to which ethyl bromacetate was added were diluted 10 times. The pH of each dilution is adjusted to 7.0 ± 0.2 and divided into 50ml parts; then one adds aseptically 0.5ml of a sterile solution of leavening extract (25gr + 100ml H_2O) and 10ml of a sterile glucose solution (50g + 100ml H_2O).

The implantation is done with 0.5ml of leaven suspension of 10^6 cells/ml. The Einhorns are filled, then placed to incubate with readings being made after 24, 30, 36, and 48 hours.

A blank run is done at the same time on a pasteurized artificial serum composed of 0.02% benzoic acid and 0.5% NaCl. The same proportions of glucose and leavening extract were added.

RESULTS

		Gaseous volume in cm^3 after:			
		24 h.	27 h.	29 h.	34 h.
45	serum + glucose + leavening extract	4.5	17.5	17.5	
46	pH 7 dilution 1:10	3	12	17.5	
47	serum + glucose + leavening extract	0	0	0	1.5
48	pH 7 + 2mg/liter ethyl bromacetate dilution 1:10	0	0	0	1.5
49	artificial serum + glucose + leavening extract	1.5	7.5	11.5	17.5
50	pH 7	1.5	7.5	12	17.5

Conclusion

The cessation of fermentation is most clear in the sample to which 2 mg of ethyl bromacetate were added per liter.

6 - Chopped Meat (8)

Experiments were done on the broths resulting from a double extraction, acid (pH 3) and alkaline (pH 8), in a way so as to extract at once the derivatives of monobromacetic acid and the various aspetic acids eventually present (benzoic and boric acids, for example).

Method of Operation

Experiments were done on: chopped pork as is and the same chopped meat to which 0.8% boric acid was added.

Acid Extraction

10 g of meat are mixed in a "Turmix" with 100 ml of a 0.5% solution of tartric acid; it is filtered, the pH is brought to 3 it is pasteurized at 80°C for 1 minute (verified with the aid of an identical receptacle containing the same quantity of liquid into which a thermometer is plonged); then we added 5 ml of a glucose solution (50 g + 100 ml) and 1 ml of a solution of leavening extract (25 g + 100 ml H_2O).

The pH is adjusted to 4 $\pm$ 0.2 aseptically.

Alkaline Extraction

10 g of meat are mixed with 100 ml of a 0.1% soda solution; it is filtered and brought to a pH of 3 and the process is continued as for the preceding extraction.

The implantation is done with 0.5 ml of leaven solution of 10^6 cells/ml. The "Einhorns" are filled, kept at 24°C and the readings are made after 24 and 30 hours.

RESULTS

		Gaseous volume in cm^3 after:	
		24 h.	30 h.
51	Chopped pork dilution 1: 10	16	17.5
52	Acid extraction	14.5	17.5
53	Chopped pork dilution 1: 10	7.5	17.5
54	Alkaline extraction	10	17.5
55	Chopped pork + 0.8% boric acid	13	17.5
56	dilution 1: 10 Acid extraction	9	17.5
57	Chopped pork + 0.8% boric acid	0	2
58	dilution 1: 10 Alkaline extraction	0	2

Conclusions

In this case, it is the alkaline extraction which is effective. Further, it proves that the concentration of the boric acid used gives a positive result.

7 - Ice Cream

A good cream ice cream represents a sufficient nutritive milieu for S. cerevisiae; therefore it is not enriched with either glucose or leavening extract.

The microbial flora being very rich in ice cream, oblige us to use a massive implantation (10^6 cells/ml in the solution to be incubated) in order to effectively combat the microbial antagonism.

Method of Operation

The experiments were done on: ice cream as is: the same ice cream to which was added 2 mg of ethyl bromacetate per liter; the same cream to which 2 mg of ethyl monobromacetate per liter was added;

In amounts of 100 ml of each sample, we implanted 1 ml of a leaven suspension containing 10^8 cells/ml. The "Einhorns" are filled and kept at 24°C.

Readings are made after 15, 18 and 20 hours.

RESULTS

		Gaseous volume in cm^3 after:	
		18 h.	20 h.
59	Ice cream	17.5	
60	as is	17.5	
61	Ice cream + 1 mg/liter	12	17.5
62	ethyl monobromacetate	13	17.5
63	Ice cream + 2 mg/liter	11.5	15
64	ethyl monobrmacetate	10.5	14.5

Conclusions

Because the massive implantations, the results were obtained after only 18 hours. An earlier reading (14 hours for example) would have given clearer results.

8 - Influence of Acetic Acid on the Growth and Fermentation Capacity of S. Cerevisiae

The problem of antiseptic research is sometimes posed with such products as marinades of semi-conserved fish and some attempts were made to

study the influence of acetic acid on the growth and fermentation capacity of S. cerevesiae.

Method of Operation

100 ml of aqueous solutions of 5% (w/w), 1% (w/w), 0.5% (w/w) acetic acid were added to 10 ml of a glucose solution (50 g + 10 ml H_2O) and 0.25 ml leavening extract (25 g + 100 ml H_2O). Half of the experiments were done without modifying the pH; for the other experiments, the pH of the 5% solution was fixed at 6.8 before diluting it.

A blank run was done at the same time on an aqueous solution containing the same concentration of glucose and leavening extract as in the experimental samples.

RESULTS

		Gaseous volume in cm^3 after:	
		24 h.	48 h.
65	H_2O + leavening extract	8.5	17.5
66	+ glucose	7.5	17.5
67	H Ac 5% + leavening extract	0	0
68	+ glucose	0	0
69	H Ac + leavening extract	0	0
70	+ glucose	0	0
71	H Ac 0.5% + leavening extract	0	0
72	+ glucose	0	0
73	H Ac 5% + leavening extract + glucose	0	0
74	pH 6.8	0	0
75	H Ac 1% + leavening extract + glucose	0	0
76	pH 6.8	0	0
77	H Ac 0.5% + leavening extract + glucose	0	0
78	pH 6.8	0	0

Conclusions

At these concentrations, acetic acid whether of natural pH or pH 6.8, has the effect of total inhibition on the fermentative capacity of S. cerevisiae.

General Conclusions

If all the results are not clearly decisive, the combination of them all enables us to make conclusions of significant interest on the microbiological method.

Beyond its real scientific value, the method shows incontestable qualities from the point of view of the simplicity of the materials and operations.

The method requires of the analyst only a relatively limited time and because of the fact that it can be applied to numerous food products, it is uniformly appealing.

If it lacks specificity, it has the great advantage of discovering from the first experiment, the presence of an effective antiseptic, no matter what its nature.

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Quelques résultats de recherche d'antiseptiques par voie microbiologique dans les denrées alimentaires (*)

Mol. Ann. Gembloux 62: 51-64, (1956)

par

L. Paix (1) et J. H. H. Mol (2).

Depuis longtemps, l'attention des hygiénistes a été attirée sur les dangers pouvant résulter de l'absorption d'antiseptiques introduits de plus en plus souvent dans les aliments (2), (3), (4).

Par ailleurs, leur nombre, leur diversité et leur efficacité allant sans cesse croissant, rendent la tâche du chimiste malaisée : comment en effet déceler d'abord, identifier ensuite la présence de substances dont la concentration est de l'ordre du milligramme par kilog. ou litre ?

Les publications du Docteur Mossel, relatives à la détection aspécifique des antiseptiques par voie microbiologique ouvrent une voie nouvelle et pleine de promesses à l'étude du problème.

La méthode employée consiste à inoculer dans le substrat convenablement préparé, une suspension de *Saccharomyces cerevisiae*, sous forme d'une simple levure de boulangerie diluée de manière appropriée. On transvase dans des tubes Einhorn que l'on place à 24° C. Après 24, 30, 48 ou 67 h. selon le cas, on mesure le volume de gaz dégagé (1).

Si le produit en question contient un antiseptique en concentration efficace, la fermentation sera freinée ou même inexistante.

Différentes denrées alimentaires ont été envisagées au cours de ce travail : la bière, la confiture, le lait, la limonade, la margarine, la viande et la glace.

Deux remarques d'ordre général :

(*) Travail effectué sous la direction de Docteur D. A. A. Mossel dans le Laboratoire du « Centraal Instituut voor Voedingsonderzoek T. N. O. » à Utrecht, Pays-Bas (Directeur Dr M. van Eekelen).

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— La sensibilité du test et la concentration de l'inoculum varient en fonction inverse ; ceci, par suite des combinaisons intervenant entre matières protéiques et antiseptiques et diminuant l'efficacité de ces derniers.

D'autre part, dans le cas de produits naturellement riches en germes, on est obligé pour lutter contre l'antagonisme microbien d'utiliser des suspensions de *S. cerevisiae* plus concentrées (10^4 cellules/ml dans la solution inoculée).

Ces deux points de vue doivent être évidemment conciliés. Il faut encore noter qu'une inoculation concentrée permet de faire des lectures plus rapides.

— Il est absolument nécessaire, quel que soit le produit en expérience, de faire un blanc, parallèlement aux essais, soit sur un substrat identique de provenance certaine, soit sur une solution artificielle.

Le pouvoir fermentatif de la levure doit être vérifié dans les conditions exactes de l'expérience : on est certain de cette manière que la souche utilisée est active, que le substrat contient suffisamment de facteurs de croissance et aucun inhibiteur naturel, et l'on échappe au danger de faux positifs.

1° La bière (5).

La bière étant en général un milieu trop pauvre pour permettre une fermentation vivante, on ajoute pour 100 ml de bière, 5 ml d'une solution stérile de glucose (50 gr + 100 ml H^2O) et 10 ml de solution nutritive de Wickerham (sels minéraux, vitamines). Celle-ci est stérilisée par filtration et stockée au réfrigérateur.

Mode opératoire.

Les essais ont été réalisés en mettant en expérience :

une bière hollandaise (connue sans antiseptique) telle quelle;

la même bière additionnée de $HgCl^2$ à raison de 5, 10, 20 mg Hg /l;

la même bière additionnée de dérivé phénylmercurique à raison de 0,5 et 1,0 mgr Hg/l;

la même bière additionnée de monobromacétate d'éthyle à raison de 0,5 mg /l;

la même bière additionnée d'actidione en concentration de 0,2 mg/l;

une « petite bière » belge telle quelle;

une bière belge spéciale telle quelle.

50 ml de chaque échantillon de bière dégazée, telle quelle ou additionnée d'antiseptique, sont enrichis sous conditions aseptiques avec 2,5 ml d'une solution de glucose (50 gr + 100 ml H^2O) et 5 ml de la solution de Wickerham.

L'inoculum est préparé sous forme d'une suspension de 1 gr de levure de boulangerie en paquet (vieille de moins de 3 jours et conservée en glacière) dilué dans 9 ml d'eau physiologique (0,85 % NaCl) stérile (cette solution contient en moyenne 10^9 cellules/ml) ; on dilue successivement en eau physiologique stérile jusqu'à obtenir une solution à 10^4 cellules/ml. De cette dernière dilution, on ensemence 0,5 ml dans chaque échantillon.

Les « Einhorn » sont remplis, placés à l'étuve à 24° C. La lecture du volume gazeux se fait après 48, 67 et 72 h.

Résultats.

	Milieu	Volume de gaz en cm³ après : 48 h	67 h	72 h
1	Bière hollandaise	0	17,5	
2	+ glucose + W. D.	0	17,5	
3	Bière hollandaise	0	0	1,5
4	+ glucose + W. D. + 5 mg Hg /l ($HgCl^2$)	0	0	1,5
5	Bière hollandaise	0	0	0
6	+ glucose + W. D. + 10 mg Hg /l ($HgCl^2$)	0	0	0
7	Bière hollandaise	0	0	0
8	+ glucose + W. D. + 20 mg Hg/l ($HgCl^2$)	0	0	0
9	Bière hollandaise	0	0	0
10	+ glucose + W. D. + 0,5 mg Hg /l (sel phénylmercurique)	0	0	0
11	Bière hollandaise	0	0	0
12	+ glucose + W. D. + 1 mg Hg /l (sel phénylmercurique)	0	0	0
13	Bière hollandaise	0	0	0
14	+ glucose + W. D. + 0,5 mg /l monobromacétate d'éthyle	0	0	0
15	Bière hollandaise	0	0	0
16	+ glucose + W. D. + 0,2 mg /l actidione	0	0	0
17	« Petite bière » belge	5	17,5	
18	+ glucose + W. D.	0	17,5	
19	Bière belge spéciale	0	0	0
20	+ glucose + W. D.	0	0	0

A chaque essai correspondent deux « Einhorn ».

Les volumes gazeux indiqués dans le tableau sont calculés en multipliant la hauteur du volume gazeux dégagé par la valeur approximative de 1,5.

Conclusions.

Tous les échantillons additionnés d'antiseptique ou d'antifongique ont donné des résultats positifs. Partant du blanc, on considère arbitrairement une inhibition de fermentation (mesurée comme volume gazeux) supérieure ou égale à 50% comme résultat positif.

La bière belge spéciale apparaît nettement contenir un inhibiteur de la fermentation. D'autre part, on y a trouvé par voie chimique une teneur en Hg de 1,7 mg /l.

Une teneur en Hg de 5 mg /l introduit sous forme de $HgCl^2$ semble une concentration se rapprochant du seuil de perceptibilité, tandis qu'une teneur de 0,5 mg /l de Hg introduit sous forme de sel phénylmercurique donne un positif net. Ceci démontre bien que le pouvoir antiseptique du Hg dépend du composé sous la forme duquel il est introduit et c'est une supériorité de la méthode microbiologique que de pouvoir mesurer l'efficacité *réelle* d'un antiseptique et cela, dans le milieu même auquel il a été additionné.

2° La confiture.

La pression osmotique régnant dans une confiture se révèle trop élevée pour l'activité de *S. cerevisiae;* c'est pourquoi une dilution de 1 à 5 est jugée nécessaire.

On supplée à la carence de la confiture diluée en facteurs de croissance en opérant la dilution 1 : 5 avec une solution stérile à 0,30 % d'extrait de levure.

Certaines confitures, principalement à base de fruits citrins, contiennent des peroxydes de terpène, inhibiteurs naturels de *S. cerevisiae.* Afin d'éviter le faux positif, on ajoute dans le « Einhorn », avant stérilisation, 1 à 1,5 gr de foie coupé en petits morceaux ; la variation du potentiel redox qui en découle permet d'échapper à cet ennui. Cependant il faut noter que la sensibilité de la méthode en est légèrement diminuée par suite de l'introduction de matières organiques.

Les échantillons ont été ajustés à pH 5,5 ± 0,1 afin d'inactiver les acides benzoïque et sulfureux d'emploi légal en Hollande.

Mode opératoire.

Les essais ont été réalisés en mettant en expérience :

une marmelade hollandaise telle quelle;
la même marmelade additionnée de foie;
la même marmelade additionnée de foie et de monobromacétate d'éthyle à raison de 1 mg/kg;
la même marmelade additionnée de foie et de monobromacétate d'éthyle à raison de 2 mg/kg.

Chaque échantillon de 50 gr de confiture telle quelle ou additionnée de monobromacétate d'éthyle est dilué avec 200 ml d'une solution à 0,30 % d'extrait de levure (stérile). On mélange au « Turmix » pendant 5', ajuste le pH à 3,5 environ, pasteurise 1' à 80° C (par immersion dans un bain d'eau à 85° C, avec comme contrôle, un récipient de même forme contenant le même volume d'eau distillée et dans lequel on plonge le thermomètre).

On ajuste ensuite le pH à 5,5 aseptiquement à l'aide de NaOH 10 %. On inocule à 50 ml de chaque essai 0,5 ml d'une suspension de levure contenant environ 10^6 cellules/ml, remplit le « Einhorn » (contenant ou non du foie suivant le cas) et met à l'étuve à 24° C.

Les lectures se font après 24, 30 et 36 h.

Résultats.

		Volume de gaz en cm³ après : 24 h	30 h
21	Eau	1,5	17,5
22	+ glucose + extrait levure	1	13
23	Confiture telle quelle	1,5	16,5
24	Dilution 1 : 5	1,5	17,5
25	Confiture telle quelle	15	17,5
26	Dilution 1 : 5 Foie dans le Einhorn	13,5	17,5
27	Confiture + 1 mg/kg monobromacétate d'éthyle	2,5	17,5
28	Dilution 1 : 5 Foie dans le Einhorn	2,5	17,5
29	Confiture + 2 mg/kg monobromacétate d'éthyle	0	5,5
30	Dilution 1 : 5 Foie dans le Einhorn	0	8,5

Conclusions.

Dans l'échantillon contenant 2 mg de monobromacétate d'éthyle au kilog., la fermentation est nettement freinée. Pour l'essai à

1 mg/kg, on ne peut rien conclure, la fermentation démarrant de la même manière que dans l'essai à blanc et l'essai de confiture telle quelle sans ajoute de foie dans le « Einhorn ».

La présence de foie donnerait une preuve nette de son efficacité si l'essai sans foie ne fermentait pas dans la même mesure que le blanc. Toutefois, il ne faut pas perdre de vue que le blanc, solution artificielle, ne contient pas autant de facteurs de croissance qu'une confiture riche en fruits et on peut se demander si l'effet de ces facteurs ne contrebalance pas efficacement celui des inhibiteurs naturels.

Dans ce cas particulier, le moment le plus favorable pour la lecture se place à 30 h d'incubation.

Au point de vue de la sensibilité, on peut remarquer que, dans le cas de confiture, l'essai contenant 2 mg d'ester monobromacétique/kg donne un résultat positif net tandis que dans le cas précédemment traité, celui de la bière, un positif encore plus net est obtenu avec seulement 0,5 mg d'ester/l. Ce résultat est prévu et normal car, dans la bière, la méthode a été mise au point de manière à reculer aussi loin que possible le seuil de perceptibilité : tout d'abord en ensemençant (le milieu s'y prête) une suspension de levure peu concentrée (10^4 cellules/ml), d'autre part en introduisant les facteurs de croissance supplémentaires sous forme de solution de Wickerham. Celle-ci présente sur l'extrait de levure outre l'avantage d'être de composition définie, celui d'éviter l'ajoute à la bière (naturellement pauvre en protéines et acides aminés à fonction thiol) de composés sulfhydrilés neutralisant l'action de certains antiseptiques et par là même abaissant la sensibilité du test.

3° Le lait (6).

Le lait, véritable milieu d'élection pour les microorganismes, ne peut être stérilisé par la chaleur avant l'essai microbiologique, certains antiseptiques comme l'ester monobromacétique et la chloropicrine ne résistant pas à un tel traitement.

Comme signalé plus haut, pour lutter contre l'antagonisme microbien, un ensemencement massif de 10^6 cellules/ml doit être utilisé dans les essais.

Le supplément hydrocarboné nécessaire dans le cas du lait, est fourni sous forme de glucose à partir d'une solution stérile (50 gr + 100 ml H_2O) dont on ajoute 5 ml pour 100 ml de lait.

L'emploi de *S. cerevisiae* présente deux avantages ici :

a) Les antibiotiques employés en thérapeutique vétérinaire n'ont pas d'action sur lui.

b) les réductions de pH (jusqu'à 4,5 au plus) fréquentes dans les laits d'été ne nuisent pas à son développement ; un tampon n'est donc pas nécessaire.

Mode opératoire.

Les essais ont porté sur :
un lait pasteurisé tel quel ;
un lait pasteurisé + 1 mg de monobromacétate d'éthyle /l ;
un lait pasteurisé + 2 mg de monobromacétate d'éthyle /l.

50 ml de chaque échantillon de lait tel quel ou additionné d'antiseptique sont enrichis stérilement avec 2,5 ml d'une solution de glucose (50 gr + 100 ml H^2O). On ensemence avec 0,5 ml d'une suspension à 10^6 cellules de levure /ml, remplit les « Einhorn » et place à l'étuve à 24° C.

Les lectures se font après 24 et 48 h.

Résultats.

		Volume de gaz en cm³ après : 24 h	40 h	48 h
31	Lait pasteurisé	coag.	17,5	
32	+ glucose	coag.	13,5	17,5
33	Lait pasteurisé	0	0	2,5
	+ glucose	0	0	2,5
34	+ 1 mg /l monobromacétate d'éthyle			
35	Lait pasteurisé	0	0	0
	+ glucose			
30	+ 2 mg /l monobromacétate d'éthyle	0	0	1

Conclusions.

Les échantillons additionnés de monobromacétate d'éthyle ne présentent aucune fermentation.

4° La limonade (7).

La remarque faite sur la confiture et concernant les inhibiteurs naturels tels que les peroxydes de terpène est également valable dans ce cas ci.

Certaines boissons vendues sous la dénomination de « limonade » sont des produits purement synthétiques à base de sucre, d'acide citrique, d'essence et de colorant artificiel ; les facteurs de croissance en étant évidemment absents, l'ajoute d'extrait de levure s'avère indispensable.

On peut se demander dans quelle mesure les colorants employés dans ce genre de boisson inhibent le développement de *S. cerevisiae*.

Le cas de l'éosine a été étudié : les quantités tolérables de brome introduit sous cette forme vont jusqu'à 50 mg Br /l ce qui représente 50 fois la quantité d'antiseptique bromé décelable.

En Hollande, les acides sulfureux et benzoïque étant également permis dans les limonades, le pH est fixé à 5,5 ± 0,1 de manière à les inactiver.

Mode opératoire.

Les essais ont porté sur :
une limonade hollandaise à base de cerises, telle quelle ;
la même limonade additionnée de 0,2 mg de monobromacétate d'éthyle /l ;
la même limonade additionnée de 0,4 mg de monobromacétate d'éthyle /l.

A 50 ml de chacun des échantillons de limonade telle quelle ou additionnée d'antiseptique, on ajoute 0,5 ml d'une solution stérile d'extrait de levure (25 gr + 100 ml H^2O) et porte le pH aseptiquement à 5,5.

Un essai à blanc est mené en parallèle sur de l'eau (pH 5,1) additionnée de 1 ml d'extrait de levure (25 gr + 100 ml H^2O) et de 5 ml de glucose (50 gr + 100 ml H^2O) pour 100 ml d'eau.

Chaque fraction de 50 ml est ensemencée avec 0,5 ml d'une suspension de levure à 10^6 cellules /ml. Les « Einhorn » sont remplis, placés à 24° C.

La lecture se fait après 24, 30 et 48 h.

Résultats.

		Volume de gaz en cm³ après : 24 h	30 h
37	Limonade	15	17,5
	+ extrait de levure		
38	pH 5,4	11,5	17,5
39	Eau	1,5	7
	+ extrait de levure		
40	+ glucose	3	5,5
	pH 5,1		
41	Limonade	1,5	4,5
	+ extrait de levure		
42	+ 0,2 mg /l	1	4
	monobromacétate d'éthyle		
	pH 5,4		
43	Limonade	0	1,5
	+ extrait de levure		
44	+ 0,4 mg /l	0	2,5
	monobromacétate d'éthyle		

Conclusions.

La lecture la plus favorable se fait après 30 h. d'incubation.

Le blanc ne fermente ni aussi vite ni aussi fort que la limonade telle quelle, mais il ne faut pas perdre de vue la richesse des fruits en facteurs de croissance ; or, il s'agit ici d'une limonade à base de jus de fruit.

Les concentrations de dérivés bromés additionnés sont nettement inhibitrices.

5° La margarine.

La recherche se faisant sur le « non-gras », les colorants liposolubles incorporés ne gênent d'aucune façon.

On a employé une dilution 1 : 10 en raison de l'influence inhibitrice du chlorure de sodium.

Mode opératoire.

Les essais ont été réalisés sur :

le « non-gras » d'une margarine dilué 1 : 10 ;

le même « non-gras » additionné de 2 mg/l de monobromacétate d'éthyle, puis dilué 1 : 10.

Une quantité suffisante de margarine est introduite dans un récipient stérile et fondue à 43 °C ; le sérum est prélevé aseptiquement.

Les échantillons de sérum tel quel ou additionné de monobromacétate d'éthyle sont dilués 10 fois. On ajuste à 7,0 ± 0,2 le pH de chaque dilution et à des fractions de 50 ml, on ajoute aseptiquement 0,5 ml d'une solution stérile d'extrait de levure (25 gr + 100 ml H^2O) et 10 ml d'une solution stérile de glucose (50 gr + 100 ml H^2O).

L'ensemencement se fait avec 0,5 ml d'une suspension de levure à 10^6 cellules/ml. Les « Einhorn » remplis sont mis à incuber, les lectures se faisant après 24, 30, 36 et 48 h.

Un essai à blanc est mené en parallèle sur un sérum artificiel pasteurisé et composé de 0,02 % d'acide benzoïque, 0,5 % de NaCl. On ajoute les mêmes proportions de glucose et d'extrait de levure qu'au sérum vrai.

Résultats.

		Volume de gaz en cm³ après : 24 h	27 h	29 h	34 h
45	sérum + glucose + extrait de levure	4,5	17,5	17,5	
46	pH 7 dilution 1 : 10	3	12	17,5	
47	sérum + glucose + extrait de levure	0	0	0	1,5
48	pH 7 + 2 mg/l monobromacétate d'éthyle dilution 1 : 10	0	0	0	1,5
49	sérum artificiel + glucose + extrait de levure	1,5	7,5	11,5	17,5
50	pH 7	1,5	7,5	12	17,5

Conclusions.

Le freinage de la fermentation est tout à fait net dans l'essai additionné de 2 mg de monobromacétate d'éthyle/l.

6° La viande hachée (8).

Les essais se font sur les liqueurs résultant d'une double extraction, acide (pH3) et alcaline (pH8), de manière à extraire à coup sûr les dérivés de l'acide monobromacétique et les divers antiseptiques acides éventuellement présents (acides borique et benzoïque p. ex.).

Mode opératoire.

Les essais portent sur :

un hachis de porc tel quel et le même hachis additionné de 0,8 % d'acide borique.

Extraction acide.

10 gr de viande sont malaxés dans un « Turmix » avec 100 ml d'une solution à 0,5 % d'acide tartrique ; on filtre, ramène le pH à 3, pasteurise à 80° C pendant 1' (en vérifiant à l'aide d'un récipient identique contenant la même quantité de liquide dans lequel plonge

un thermomètre), ajoute aseptiquement 5 ml d'une solution de glucose (50 gr + 100 ml) et 1 ml d'une solution d'extrait de levure (25 gr + 100 ml H^2O).

Le pH est ajusté à 4 ± 0,2, aseptiquement.

Extraction alcaline.

10 gr de viande sont malaxés avec 100 ml d'une solution de soude à 0,1 %, on filtre, ramène le pH à 3 et continue comme pour l'extrait précédent.

L'ensemencement se fait avec 0,5 ml d'une suspension de levure à 10^6 cellules/ml. Les « Einhorn » sont remplis, mis à 24° C, la lecture se fait après 24 et 30 h.

Résultats.

		Volume de gaz en cm³ après : 24 h	30 h
51	Hachis de porc dilution 1 : 10	16	17,5
52	Extraction acide	14,5	17,5
53	Hachis de porc dilution 1 : 10	7,5	17,5
54	Extraction alcaline	10	17,5
55	Hachis de porc + 0,8 % acide borique	13	17,5
56	dilution 1 : 10 Extraction acide	9	17,5
57	Hachis de porc + 0,8 % acide borique	0	2
58	dilution 1 : 10 Extraction alcaline	0	2

Conclusions.

Dans ce cas-ci, c'est l'extraction alcaline qui est efficace. D'autre part, il est prouvé que la concentration d'acide borique utilisée donne un positif net.

7° La glace.

Une bonne crème glacée représente un milieu nutritif suffisant pour *S. cerevisiae* : elle n'est donc enrichie ni de glucose ni d'extrait de levure.

La flore microbienne fort riche dans une glace, oblige à employer un ensemencement massif (10^6 cellules/ml dans la solution à

incuber) de manière à lutter efficacement contre l'antagonisme microbien.

Mode opératoire.

Les essais ont été faits sur :

une crème glacée telle quelle ;

la même crème additionnée de 1 mg de monobromacétate d'éthyle/l ;

la même crème additionnée de 2 mg de monobromacétate d'éthyle/l ;

Dans des portions de 100 ml de chaque échantillon, on ensemence 1 ml d'une suspension de levure contenant 10^8 cellules/ml. Les « Einhorn » sont remplis, mis à 24° C.

Les lectures sont faites après 15, 18 et 20 h.

Résultats.

		Volume de gaz en cm³ après : 18 h	20 h
50	Glace	17,5	
60	telle quelle	17,5	
61	Glace + 1 mg l	12	17,5
62	monobromacétate d'éthyle	13	17,5
63	Glace + 2 mg l	11,5	15
64	monobromacétate d'éthyle	10,5	14,5

Conclusions.

En raison de l'ensemencement massif, les résultats sont obtenus après 18 heures seulement. Une lecture plus rapide (14 h par exemple) aurait donné des résultats plus nets.

8° Influence de l'acide acétique sur la croissance et le pouvoir fermentatif de S. cerevisiae.

Le problème de la recherche d'antiseptiques se posant parfois au sujet de produits tels que les marinades de poissons semi-conservés, quelques essais ont été faits afin d'étudier l'influence de l'acide acétique sur la croissance et le pouvoir fermentatif de *S. cerevisiae*.

Mode opératoire.

100 ml de solutions aqueuses à 5 % (w/w), 1 % (w/w), 0,5 % (w/w) d'acide acétique ont été additionnées de 10 ml de solution

de glucose (50 gr + 10 ml H^2O) et de 0,25 ml d'extrait de levure (25 gr + 100 ml H^2O). La moitié des essais ont été faits sans modifier le pH ; pour les autres essais on a fixé à 6,8 le pH de la solution 5 % avant de la diluer.

Un « blanc » a été mené en parallèle sur une solution aqueuse contenant la même concentration de glucose et d'extrait de levure que les essais.

Résultats.

		Volume de gaz en cm³ après : 24 h	48 h
65	H^2O	8,5	17,5
66	+ extrait de levure + glucose	7,5	17,5
67	H Ac 5 %	0	0
68	+ extrait de levure + glucose	0	0
69	H Ac 1 %	0	0
70	+ extrait de levure + glucose	0	0
71	H Ac 0,5 %	0	0
72	+ extrait de levure + glucose	0	0
73	H Ac 5 % + extrait de levure + glucose	0	0
74	pH 6,8	0	0
75	H Ac 1 % + extrait de levure + glucose	0	0
76	pH 6,8	0	0
77	H Ac 0,5 % + extrait de levure + glucose	0	0
78	pH 6,8	0	0

Conclusions.

A ces concentrations, l'acide acétique, que ce soit au pH naturel ou au pH 6,8, a un effet d'inhibition totale sur le pouvoir fermentatif de *S. cerevisiae*.

Conclusion générale.

Si tous ces résultats ne sont pas d'une netteté décisive, leur ensemble permet cependant de conclure au très grand intérêt de la méthode microbiologique.

Outre sa valeur scientifique réelle, elle présente des qualités incontestables au point de vue de la simplicité du matériel et des manipulations.

La méthode n'exige de l'analyste qu'un temps relativement restreint et, du fait qu'elle s'applique à de très nombreux produits alimentaires, possède un caractère standard séduisant.

Si elle manque de spécificité, elle a le grand avantage de dépister dès le premier essai, la présence d'un antiseptique efficace, quelle que soit sa nature.

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SURVIVAL OF *SALMONELLA TYPHIMURIUM* IN COLD-PACK CHEESE FOOD DURING REFRIGERATED STORAGE[1]

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ABSTRACT

Fourteen batches of cold-pack cheese food were contaminated with *Salmonella typhimurium* during manufacture. Cheese food stored at 4.4 and 12.8 C was tested at weekly intervals and salmonellae were enumerated by means of a Most Probable Number technique. A rapid decline in number of salmonellae occurred during the first week of storage regardless of temperature or composition of the product. Survival beyond this time was more closely related to both conditions. Viable salmonellae could not be recovered, after 3 weeks at 12.8 C or 5 weeks at 4.4 C, from cheese food adjusted to pH 5.0 with lactic acid and fortified with 0.24% potassium sorbate. Substituting sodium propionate for sorbate resulted in 14 and 16 weeks of survival by salmonellae when cheese food was held at 12.8 and 4.4 C, respectively. Partial or complete replacement of lactic acid by acetic acid was accompanied by somewhat longer survival of salmonellae than when only lactic acid was used. Elimination of added acid from the cheese food resulted in survival of salmonellae for 6 and 7 weeks when potassium sorbate was present, for 16 and 19 weeks when sodium propionate was used, and in excess of 27 weeks when no preservative was added.

Salmonellae have been recognized as human pathogens for nearly 100 years. Salmonellosis is primarily an animal disease; human food-borne illness is a more or less accidental consequence of animal infection. Food-borne salmonellae are most often associated with poultry, poultry products, meat, and meat products. It is estimated that each year nearly two million Americans suffer from salmonellosis (3, 7). Although the disease frequently is mild, approximately 100 persons in the United States die from salmonellosis each year and the annual economic loss attributed to this problem ranges from 10 to 100 million dollars (13).

Milk once was a common vehicle for the dissemination of salmonellae. Since pasteurization has become common, milk has only rarely been incriminated in outbreaks of salmonellosis. However, nonfat dry milk has recently been associated with some outbreaks of salmonellosis caused by *Salmonella newbrunswick* (9). Other salmonellae also have been recovered from this product since the onset, in 1966, of a vigorous testing program (9). During 1969 the following serotypes were found in samples of commercial nonfat dry milk: *Salmonella cubana*, *Salmonella minnecapolis*, *Salmonella newington*, *Salmonella albany*, *Salmonella anatum*, *Salmonella tennessee*, and *Salmonella senftenberg* (16).

According to a recent review by Marth (9), outbreaks of salmonellosis attributable to cheese were reported as early as 1923 when, in Great Britain, persons contracted paratyphoid fever from consumption of contaminated cream cheese. Since then salmonellosis, nearly always caused by *Salmonella typhimurium* or *Salmonella typhi*, has been associated with Cheddar, Colby, Camembert, Romano Dolce, Teleme, Jack, Quarg, Cream, Cottage, and some other cheeses (9). Persons handling milk used for cheese making or the milk itself most often served as sources of the salmonellae. In some instances cheese was consumed before it was aged sufficiently long to insure inactivation of salmonellae. Although cheese made from raw milk must be ripened at not less than 1.67 C (35 F) for at least 60 days prior to sale, there is ample evidence that this treatment will not eliminate viable salmonellae, if present, from cheese (1, 5, 6, 12, 15). In the United States approximately 35% of the ripened cheese produced is used to manufacture processed cheese products of all types including cold-pack cheese food (8).

Cold-pack cheese food is prepared from cheese, nonfat dry milk, dried whey, and some other ingredients. It is possible that the finished product could be contaminated with salmonellae by some of the ingredients. In addition to cheese, where extended survival of these organisms has been reported (5, 6, 12), and nonfat dry milk, cheese food could be contaminated by dried whey which, on occasion has been found to contain salmonellae (16). Contamination of a finished product with salmonellae from ingredients has been observed in other foods (3). Additionally, cold-pack cheese food could be contaminated by personnel who are engaged in its production. Presence of salmonellae in this product

[1] Published with the approval of the Director of the Research Division, College of Agricultural and Life Sciences, University of Wisconsin.

TABLE 1. COMPOSITION OF EXPERIMENTAL BATCHES OF COLD-PACK CHEESE FOOD[1]

Ingredient	Experimental product 1	2	3	4	5	6	7
Cheddar cheese (g)	1698	1740	1740	1740	2112	1740	1740
NFDM (g)	100	100	100	100	125	100	100
Dried whey (g)	100	100	100	100	125	100	100
Butter (g)	76.2	76.2	76.2	76.2	95.1	76.2	76.2
Potassium sorbate (g)	5.4	–	5.4	5.4	6.8	–	–
Sodium propionate (g)	–	4.5	–	–	–	4.5	–
Lactic acid (ml)	8.5	10.5	7.0	–	–	–	–
Acetic acid (ml)	–	–	2.1	6.3	–	–	–
Water (ml)	241	261	261	261	327	261	261
Moisture (%)	41.81	43.36	43.65	43.73	43.58	43.49	43.24
Fat (%)	24.88	23.57	24.36	24.44	24.38	24.07	25.22

[1]Percentages of moisture and fat are average values obtained from tests on duplicate batches of each type of cheese food.

is of special significance because it receives no heat treatment nor is any additional aging required before it is consumed.

Since no information was available on the behavior of salmonellae in cold-pack cheese food, experiments were initiated to determine how long the organisms persist in such products when made according to different formulae and when stored at refrigeration temperatures. This paper reports results of the investigation. A preliminary report of this work has been given *(11)*.

MATERIALS AND METHODS

Culture

A 24 hr old nutrient broth culture of S. *typhimurium* (Department of Bacteriology, University of Wisconsin) was used to contaminate cold-pack cheese food during its manufacture. Sufficient culture was added to provide approximately 200 salmonellae per gram of product although this value was not attained in all experiments. The culture of S. *typhimurium* was maintained by daily transfer in nutrient broth.

Manufacture of cheese food and sampling procedure

The formulae used to prepare cheese food are given in Table 1. Two batches of cheese food were prepared according to each formula and data reported in this paper are average values obtained from tests on both similar batches of product. Cheddar cheese (6 to 10 months old) was ground using a hand-operated meat grinder after which the following ingredients were added: butter (melted in sterilized warm water prior to use), nonfat dry milk, dried whey, preservative (potassium sorbate or sodium propionate blended with dry ingredients before adding to the remainder of the product), and acid (lactic or acetic acid diluted with sterile water) when the pH of the product was adjusted. All ingredients were thoroughly mixed by hand (with sanitized rubber gloves in place) and with the aid of a potato ricer. Finally S. *typhimurium* (diluted with sterile buffered water just prior to use) was added and the mixing process just described was repeated. Preliminary trials with *Serratia marcescens* demonstrated that the mixing process was adequate to insure uniform distribution of added bacteria in the cheese food. Cheese food was then filled into 2 oz plastic containers fitted with screw caps and was stored at 4.4 and 12.8 C. The product was tested initially for moisture and fat contents, pH, and number of viable salmonellae. Stored cheese food was examined at weekly intervals for its pH value and number of viable salmonellae.

Federal standards for cold-pack cheese food require that the product contains a minimum of 23% butterfat and a maximum of 44% moisture. Additionally, use of 0.20% sorbic acid or 0.30% sodium propionate is permitted. All cheese food prepared in these trials complied with the indicated standards.

Fat, moisture, and pH determinations

The fat content of cheese food was determined by the modified Babcock procedure as described by Van Slyke and Price *(17)*. Moisture in the product was determined by drying 3 g cheese food in a 50 ml beaker at 110 C for 16 hr in a forced draft oven. The pH value was measured with a saturated calomel half-cell, gold electrode, and a Leeds and Northrup portable potentiometer.

Enumeration of salmonellae

Salmonellae in cheese food were enumerated by means of the Most Probable Number (MPN) technique. Each value for a given batch of cheese represents the average of results obtained by testing three samples. Consequently, each value reported in the figures represents the average of 6 samples, three from each of two batches of cheese food. Twenty grams of cheese food was blended (4 min in a Waring blendor) with 180 ml of a sterile 2% sodium citrate solution previously cooled to 5 C. Subsequent dilutions were made with the sodium citrate solution.

After blending, 1 ml aliquots of appropriate dilutions were transferred to tubes each of which contained 9 ml of brain heart infusion broth (Difco). After overnight incubation at 37 C, 1 ml quantities were transferred to tubes containing 9 ml each of selenite-cystine broth (Difco). These cultures were incubated at 37 C for 24 hr and a sample from each tube was then streaked on SS agar (Difco) modified by adding 10 g sucrose (to screen for sucrose fermenting organisms) and 6.5 g agar (to facilitate streaking) per liter. When used as described, SS agar recovered essentially only salmonellae from cheese food. This, perhaps, is more readily understood when it is realized the 6-10 month old Cheddar cheese and the other ingredients used are not likely to contain many

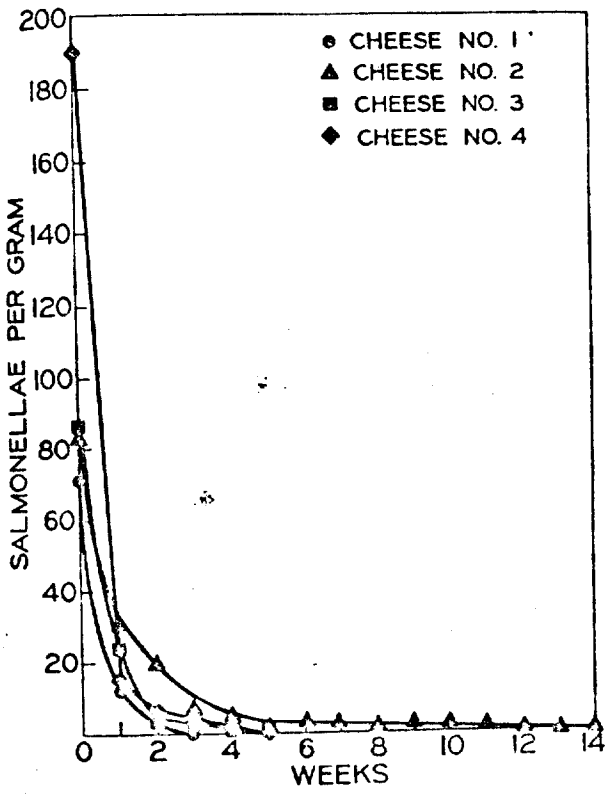

Figure 1. Survival of *Salmonella typhimurium* in cold-pack cheese food adjusted to pH 5.0, fortified with preservative, and stored at 12.8 C. Details on composition of each cheese food are in Table I.

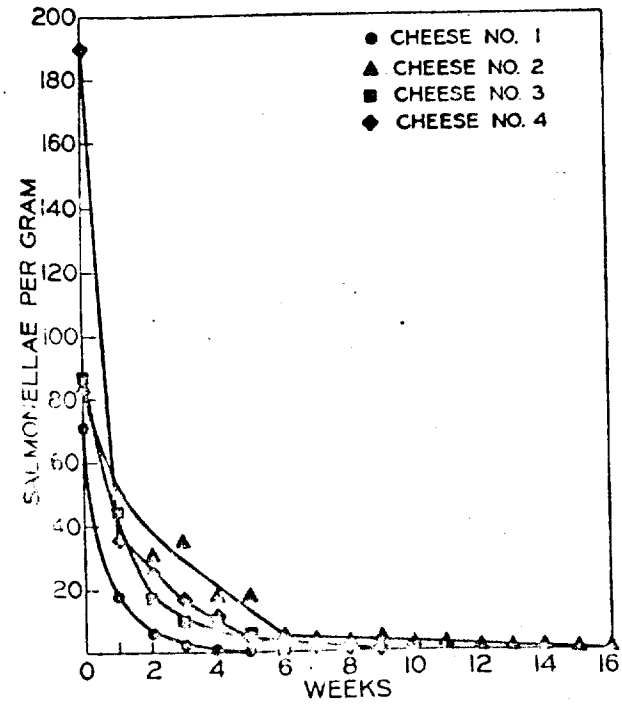

Figure 2. Survival of *Salmonella typhimurium* in cold-pack cheese food adjusted to pH 5.0, fortified with preservative, and stored at 4.4 C. Details on composition of each cheese food are in Table I.

of the organisms which normally cause difficulty in recovery of salmonellae. Plates were examined after 24 hr at 37 C and typical *Salmonella* colonies were streaked onto and stabbed into triple sugar iron agar slants. These slants were incubated 24 hr at 37 C and checked to insure that the reaction was typical of that produced by salmonellae.

RESULTS AND DISCUSSION

Survival of salmonellae in cheese food with added acid and preservative

Figures 1 and 2 present data on the survival of salmonellae in cold-pack cheese food adjusted to pH 5.0 with lactic and/or acetic acid and fortified with either potassium sorbate or sodium propionate. Data on the pH values of these products during storage are recorded in Fig. 5 and 6.

It is evident that the number of viable salmonellae in all four types of cheese food declined most rapidly during the first week of storage at both temperatures (4.4 and 12.8 C). A less precipitous decline continued during subsequent weeks of storage. Salmonellae in cheese food containing both lactic acid and potassium sorbate declined to nondetectable levels in 3 and 5 weeks when the product was held at 12.8 and 4.4 C, respectively. In contrast, 14 and 16 weeks at the same temperatures were required before salmonellae could not be recovered from cheese food made with sodium propionate instead of potassium sorbate.

Although potassium sorbate is commonly thought to be more inhibitory to yeasts and molds than is sodium propionate *(10)*, Doell *(2)* reported that the chemical at a concentration of 0.1% and at pH values of 5.0 and 6.0 was bacteriostatic to some salmonellae. A bactericidal effect was not noted under the same conditions during a 48 hr incubation period. Doell did observe inactivation of salmonellae in 24 or 48 hr at pH 5.0 when the concentration of sorbate was increased to 1.0%. Extended storage in these experiments may have accomplished what the increase in concentration did in the tests by Doell.

Replacement of lactic acid, completely or in part, with acetic acid failed to enhance destruction of salmonellae in cheese food. In fact, the organisms persisted for 4 and 5 weeks at 12.8 C and for 6 and 9 weeks at 4.4 C. The longer periods of survival just indicated were noted when acetic acid only was used and the shorter times when a mixture of lactic and acetic acid was added. Even though acetic acid apparently had no beneficial effect over lactic acid, salmonellae in cheese foods containing acetic acid and sorbate (No. 3 and 4) remained viable for approximately one-third to one-half as long as those in cheese which contained lactic acid and sodium propionate.

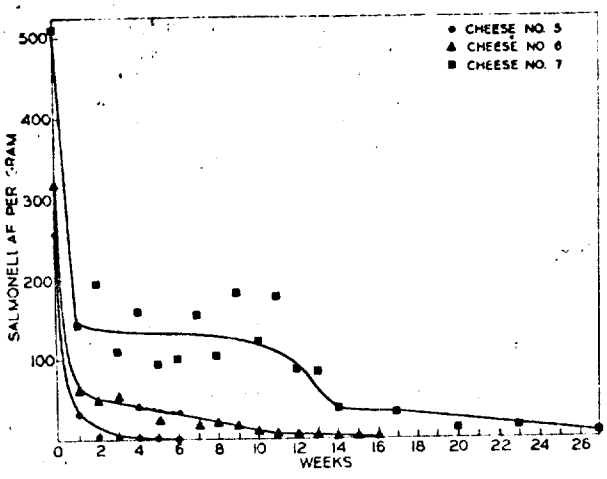

Figure 3. Survival of *Salmonella typhimurium* during storage at 12.8 C in cold-pack cheese food made without pH adjustment and with and without preservative. Details on composition of each cheese food are in Table 1.

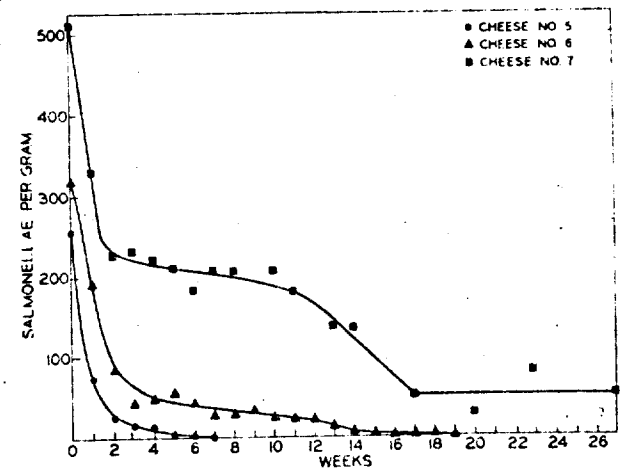

Figure. 4. Survival of *Salmonella typhimurium* during storage at 4.4 C in cold-pack cheese food made without pH adjustment and with and without preservative. Details on composition of each cheese food are in Table 1.

Several reports *(4, 9, 14, 18)* have indicated that organic acids are inhibitory to salmonellae. Propionic and acetic acids are usually thought to be more active than citric and lactic acids. Recently Subramanian and Marth *(14)* observed that citric acid retarded growth of S. *typhimurium* more than did lactic or hydrochloric acids. Wethington and Fabian *(18)* noted that *Salmonella schottmuelleri*, S. *typhimurium*, *Salmonella paratyphi*, *Salmonella enteritidis*, *Salmonella choleraesius*, and *Salmonella pullorum* survived for 144, 144, 156, 156, 156, and 132 hr, respectively, in mayonnaise made to contain 0.15% acetic acid (pH 5.0) and held at room temperature. Goepfert et al. *(5)* reported that the presence of 0.1% acetate in skimmilk at pH 4.9 enhanced inactivation of S. *typhimurium* and they suggested that accumulation of this acid during ripening of Cheddar cheese might contribute to the demise of salmonellae in cheese. In contrast to this, Hargrove et al. *(6)* claimed that acetic acid had no apparent effect greater than that of other acids on survival of salmonellae in Cheddar cheese made by direct acidification. Differences in the effect of acetic acid on salmonellae, including those reported in this paper, noted by various investigators are probably attributable to different environmental conditions which existed and which concurrent with acetic acid, affected the bacteria.

Data in Fig. 5 and 6 reveal that the pH of cheese food held at 12.8 C tended to decline during storage, whereas, at 4.4 C it remained rather constant. Undoubtedly this change in pH contributed to the more rapid disappearance of viable salmonellae from cheese food stored at the higher temperature.

Survival of salmonellae in cheese food with added preservative

Figures 3 and 4 provide data on the disappearance of viable salmonellae from cold-pack cheese food made without adjusting the pH but with added potassium sorbate (No. 5) or sodium propionate (No. 6). Detectable salmonellae persisted for 6 and 7 weeks at 12.8 and 4.4 C, respectively, when cheese food contained potassium sorbate and for 16 and 19 weeks at the same temperatures when sodium propionate was used. The pH values of cheese foods ranged between 5.20 and 5.26 when the products were prepared. During storage at 4.4 C, the pH of cheese food with potassium sorbate dropped by approximately 0.1 unit but failed to reach a pH value of 5.0 as existed in acidified products. The pH of the product with propionate declined gradually and

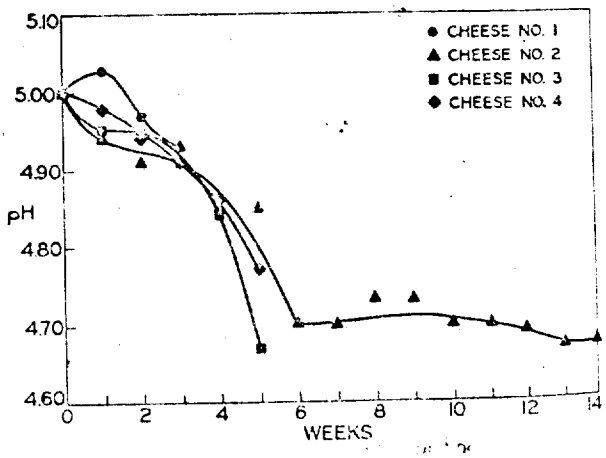

Figure 5. The pH values of acidified cold-pack cheese food containing preservatives and stored at 12.8 C.

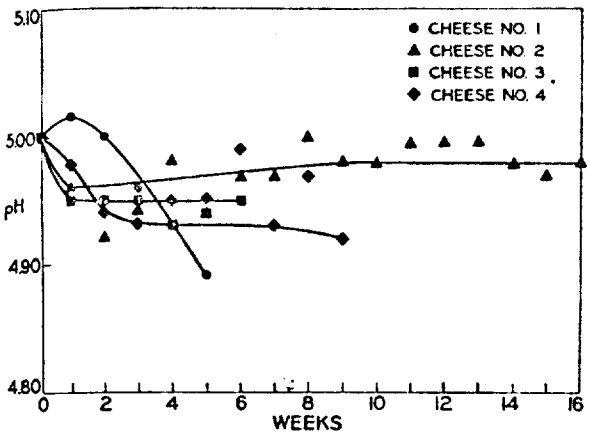

Figure 6. The pH values of acidified cold-pack cheese food containing preservatives and stored at 4.4 C.

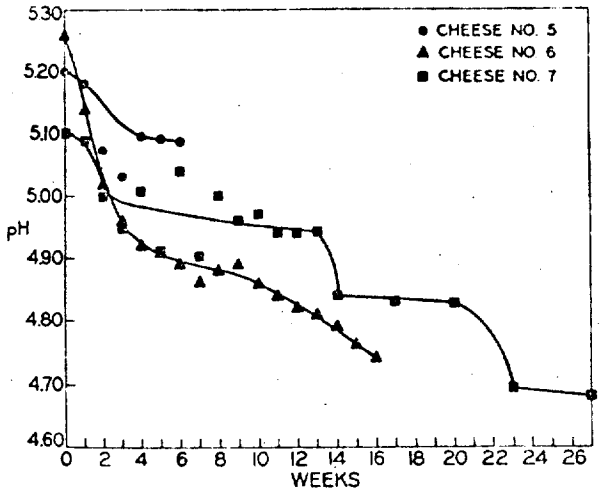

Figure 7. The pH values of cold-pack cheese food made without added acid, with or without preservatives, and stored at 12.8 C.

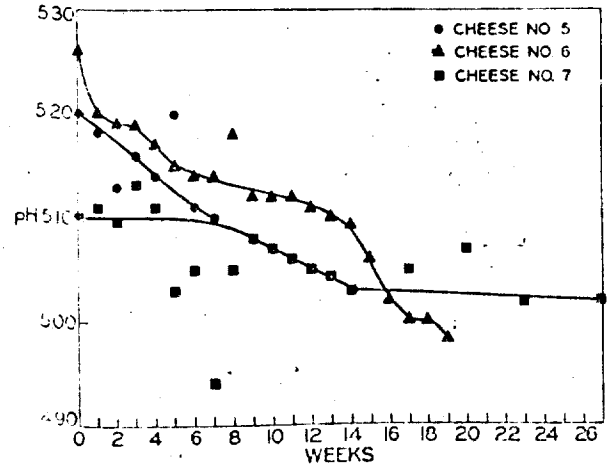

Figure 8. The pH values of cold-pack cheese food made without added acid, with or without preservative, and stored at 4.4 C.

a value of 5.0 was attained after 17 weeks of storage. Undoubtedly the higher pH of cheese food contributed to extended survival of salmonellae in the product held at 4.4 C. The decline in pH was greater in cheese food held at 12.8 C and this is probably related to more rapid demise of salmonellae from the product.

Survival of salmonellae in cheese food free of added acid or preservative

Data in Fig. 3 and 4 (No. 7) indicate that viable salmonellae remained in unfortified cheese food after 27 weeks of storage at either 12.8 or 4.4 C. A somewhat higher population appeared in cheese food stored at the lower temperature. The pH value of the product held at 4.4 C remained above 5.0 throughout storage, whereas cheese food at 12.8 C attained a pH value of 5.0 after two weeks of holding and then continued to decline below 4.7 after 23 weeks. In spite of this drop in pH, viable salmonellae persisted for at least 27 weeks. This suggests that in a product as complex as cheese food, pH (within the range associated with the product) is only one factor which governs survival of salmonellae.

Observations made in these experiments emphasize that cold-pack cheese food must be prepared from high quality ingredients which are free of hazardous microorganisms and the product must be handled to preclude contamination by such microorganisms during its manufacture. If present, salmonellae could survive in the product until it reaches the consumer, particularly if the pH of cheese food is not adjusted and if potassium sorbate is not used.

ACKNOWLEDGMENT

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Utilization of salts of volatile fatty acids by growing sheep

5.* Effects of type of fermentation of the basal diet on the utilization of salts of acetic acid for body gains

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1. An experiment is reported in which groups of six lambs were fed two basal diets supplemented at three levels with a mixture of sodium and calcium acetates.

2. The basal diets were given in amounts that provided equal intakes of digestible organic matter and crude protein. One contained 85% of concentrates (Hc), the other 40% of concentrates (Lc). Rumen contents from a sheep receiving diet Hc contained a lower molar proportion of acetate and higher proportions of propionate and butyrate than when diet Lc was given.

3. The calculated metabolizable energy of the basal Hc diet was utilized more efficiently than that of the basal Lc diet, for promoting empty body-weight and carcass-weight gains.

4. On both basal diets, lambs receiving the diets supplemented with acetate made greater live-weight, empty body-weight and carcass-weight gains than lambs given unsupplemented diets. The responses of weight gain to increasing levels of acetate were linear.

5. The responses to acetate were greater when it was given with the Hc diet than with the Lc diet. This effect was most marked for live-weight gain ($P < 0.001$), intermediate for empty body-weight gain ($P < 0.05$), but not significant for carcass-weight gain. This order of effects was in part due to a greater weight of alimentary tract tissue, and its contents, in lambs fed the Hc diet supplemented with acetate.

6. It is concluded that under certain circumstances the energy of acetate may be utilized less efficiently than energy from propionate or butyrate.

Conflicting results have been recorded from experiments in which the utilization of acetate for growth has been compared with that of propionate or butyrate. Armstrong & Blaxter (1957) and Armstrong, Blaxter, Graham & Wainman (1958) found that when acetic acid was infused into the rumen of sheep, its utilization for lipogenesis was less than that of propionic or butyric acid. Poor utilization was associated with a high heat increment, some of the reasons for which were indicated by McClymont (1953).

In previous experiments of this series where salts of volatile fatty acids (VFA) were fed to growing lambs, Orskov & Allen (1966*a*, *b*, *c*) and Orskov, Hovell & Allen (1966) found no differences in the efficiency of utilization of energy from salts of different acids. In one experiment (Orskov & Allen, 1966*c*) a high-roughage diet promoted lower weight gains than a high-concentrate diet; associated with this effect was a greater molar proportion of acetic acid in the rumen liquor of lambs fed the high-roughage diet. It was postulated that the poorer utilization of energy in this experiment might be associated with losses of energy in the formation of acetic acid rather than in its subsequent utilization.

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In the experiment reported here we studied the utilization of salts of acetic acid, added at three levels, to two basal diets which were known to induce different patterns of rumen fermentation. Addition of acetate at three levels made it possible to apply the more refined statistical method of linear regression analysis to the experimental results.

EXPERIMENTAL

Animals and facilities

Fifty-four Clun Forest and Suffolk × Clun Forest wether lambs were weaned off grass at 13–15 weeks of age when they had a mean live weight of 28 kg. They were housed in individual pens with self-cleaning floors in a building with forced ventilation.

Design and treatments

After a period of 4 weeks during which the lambs were accustomed to dry feed, they were grouped into six blocks, each block containing nine lambs of similar live weight. Within each block, lambs were allocated at random to nine treatments as follows: initial slaughter group; basal high concentrate (Hc) with (15% hay and 85% concentrate); Hc + 228 kcal acetate/d; Hc + 456 kcal acetate/d; Hc + 684 kcal acetate/d; basal low concentrate (Lc) (60% hay and 40% concentrate); Lc + 228 kcal acetate/d; Lc + 456 kcal acetate/d and Lc + 684 kcal acetate/d.

Digestibility trials

Before the main feeding experiment, the digestible organic-matter contents of the hay and the two basal concentrates were determined in vivo. Eight mature wethers were harnessed, fitted with faecal collection bags and housed in digestibility crates.

Table 1. *Digestibility of organic matter (%) in the two diets, determined with four sheep for the complete diet and with eight sheep for the hay*

	Diet Hc		Diet Lc	
	% by weight	Organic matter digestibility (%)	% by weight	Organic matter digestibility (%)
Hay	15	71·9	60	71·9
Concentrate	85	(88·2)*	40	(82·6)*
Complete diet	—	85·7	—	76·3

Ratio of digestibilities Hc to Lc = 1 to 1·124.

* Calculated from the digestibility of the hay and the complete diet.

They were first fed the hay, and subsequently each basal diet was fed to a group of four wethers. The diet under test was always fed for a preliminary period of 10 d and a collection period of 7 d. Faeces were dried to constant weight at 100° and the organic-matter contents of the feeds and faeces were determined by the method listed in the Fertilizer and Feedingstuffs Regulations (Great Britain: Parliament, 1960). The results are presented in Table 1.

The results of the digestibility trial were used to calculate the level of feeding of each basal diet which would provide equal intakes of digestible organic matter.

Composition and preparation of diets

The hay was of good quality, being made from a predominantly perennial ryegrass sward, cut at the flowering stage. It was chopped into approximately 2·5 cm lengths before feeding.

The basal diets were fed in quantities sufficient to provide equal intakes of digestible organic matter. Having decided on these quantities, the level of crude protein in each concentrate was adjusted so that the lambs also had equal intakes of crude protein. The composition of these concentrates is shown in Table 2. The rather high crude protein content of 18% was adopted so that liberal quantities of protein would be available to balance the supplementary energy derived from acetate.

Table 2. *Ingredients of two concentrates* (%) *fed in conjunction with hay to create a high-concentrate diet* (*Hc*) *or a low-concentrate diet* (*Lc*)

	Concentrate for Hc diet	Concentrate for Lc diet
Ground flaked maize	50	—
Ground barley meal	15	48
Decorticated groundnut meal	15	20
White fish meal	10	22
Molasses meal	8	8
Minerals*	2	2

4×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D were added per 1000 kg.

* Proprietary mineral mixture, without added copper.

Acetate salts were incorporated into the concentrates. To minimize the possible adverse effects of a single metallic ion, a 1 to 1 weight for weight mixture of sodium and calcium acetate was used. The amount of acetate incorporated into the concentrates was verified by chemical analysis.

All the concentrates were cubed using a 1·5 cm die, although cubing was difficult with those concentrates containing high levels of acetate.

Management of lambs

The rations were weighed daily, approximately two-thirds being offered at 17.00 hours and the remainder at 09.00 hours. Uneaten food, if any, was removed daily and dried to constant weight. Most food refusals occurred soon after the start, so that it was possible to give the lambs an equivalent amount of food later in the experiment, by making additions to the daily ration. Water was offered *ad lib.*

The lambs were weighed to the nearest 0·25 kg on 3 consecutive d in alternate weeks, and mean live weights were calculated. At this time, the food allowances for lambs in each block were recalculated, according to the adjusted live weight of the control lamb which received the basal Lc diet. The adjusted live weight was computed by adding the mean gain of all the control lambs to the live weight of the control

lamb in each block which had been recorded 2 weeks previously. This adjustment reduced the immediate effect of abnormal weight gains by an individual lamb.

To achieve a moderate growth rate in unsupplemented lambs, a level of feeding equivalent to 113 g of basal Lc diet per 4·5 kg live weight was chosen. Lambs receiving supplemented diets were offered an amount which contained the same amount of basal concentrate in addition to the acetate supplement. Over the whole experimental period the average intake of the basal Lc diet was 775 g/d. Calculated mean daily intakes of metabolizable energy from the basal diets and acetate supplements are shown in Table 3.

Table 3. *Mean daily intakes of metabolizable energy* (*ME*) (*kcal/d*) *calculated from the intended levels of feeding to groups of six lambs receiving a high-concentrate* (*Hc*) *or a low-concentrate* (*Lc*) *diet, with or without acetate supplementation*

Diet	Hay*	Concentrate†	Acetate‡	Total
Hc unsupplemented	228	1619	—	1847
Hc+228 kcal acetate/d	228	1619	228	2075
Hc+456 kcal acetate/d	228	1619	456	2303
Hc+684 kcal acetate/d	228	1619	684	2531
Lc unsupplemented	1032	815	—	1847
Lc+228 kcal acetate/d	1032	815	228	2075
Lc+456 kcal acetate/d	1032	815	456	2303
Lc+684 kcal acetate/d	1032	815	684	2531

* Hay assumed to contain 2·22 Mcal ME/kg fresh matter.
† Concentrates assumed to contain 2·76 (Hc) or 2·63 (Lc) Mcal ME/kg fresh matter.
‡ ME of acetate assumed to be heat of combustion.

The health of the lambs was generally good. One suffered from foot rot on two occasions, but in both instances the condition responded to treatment with a tincture of chloromycetin. Some mild scouring occurred which proved to be contagious; those lambs receiving large supplements of acetate appeared to be particularly prone to infection. The scouring was controlled following a subcutaneous injection of 10 ml of 33·3% (w/v) sodium sulphadimidine solution (Day, Son & Hewitt Ltd).

Slaughter procedure

The initial slaughter group was slaughtered at the beginning of the experiment, the remaining groups being slaughtered over a period of 3 d after 98, 99 and 100 d on experiment. Final live weight was taken as the average of weights recorded on 3 consecutive d immediately before slaughter. Empty body-weight was calculated by subtracting the weight of the contents of the alimentary tract from the live weight recorded just before slaughter. The empty body-weight measure avoids a biased live-weight assessment caused by different weights of alimentary tract contents, which may arise when the diets differ from each other in terms of digestibility, level of intake and rate of passage through the alimentary tract. Carcass-weights were recorded after chilling for 24 h at 4°. The carcasses were graded by an official grader of the Ministry of Agriculture, Fisheries and Food.

Rumen fermentation studies

A mature wether fitted with a rumen cannula was used to assess the pattern of rumen fermentation resulting from feeding each of the basal diets and the Lc + 684 kcal acetate/d diet.

The level of feeding was the same as that of the experimental lambs, and each diet was given for 25 d. On the 21st, 23rd and 25th days, strained samples of rumen liquor, approximately 50 ml, were taken, using a hand-operated suction pump. The rumen was sampled on four occasions during the day, at 09.00 hours (before feeding), 11.00, 14.00 and 17.00 hours (before feeding). Immediately after removal of the sample, pH was recorded to 0·01 units. Each day samples were bulked, taking 10 ml on each sampling occasion, and stored at −10°. The bulked samples were analysed for total VFA by the method of McAnally (1944) and for the proportions of VFA by the method of Youssef & Allen (1966). The results are summarized in Table 4.

Table 4. *Molar percentage of volatile fatty acids (VFA) in rumen liquor samples taken from one sheep receiving high-concentrate (Hc), low-concentrate (Lc) or low-concentrate + 684 kcal/d (Lc + 684 kcal acetate/d) diets*

(Mean values for 24 h)

	Hc	Lc	Lc + 684 kcal acetate/d
Total VFA (m-equiv./100 ml)	10·12	10·33	13·07
Acetic acid (molar %)	64·02	73·74	80·81
Propionic acid (molar %)	20·91	14·24	10·37
Butyric acid (molar %)	12·38	9·95	7·28
Valeric acid (molar %)	2·69	2·06	1·54
pH	6·50	6·76	7·09

RESULTS

Values for the initial slaughter group were used to compute average values for the relationships between live weight and empty body-weight, and between live weight and carcass weight at the start of the experiment. Consequently the empty body-weight and carcass-weight gains made during the experimental period could be estimated. These are shown in Table 5.

In the statistical analysis, results for four lambs were discarded, and a missing plot technique was used to calculate substitute values. Three of these lambs had consistently refused part of the daily ration. One lamb was on Hc + 684 kcal acetate/d treatment and refused concentrate equivalent to 22% of the intended dry-matter intake and 26% of acetate supplement, the other lambs were on Lc + 456 kcal acetate/d treatments and refused hay equivalent to 7 and 16% of the intended dry-matter intake. A fourth lamb (Hc + 228 kcal acetate/d), although consuming the diet offered, developed a progressive debility for which no specific cause was diagnosed.

In the general analysis of variance of the live-weight, empty body-weight and carcass-weight gains made during the experimental period, the responses to acetate in terms of increased weight gains were shown to be highly significant ($P < 0.001$).

The standard error of this analysis was applied to the mean gains of the two groups of lambs fed the basal unsupplemented diets Hc and Lc. With both diets the live-weight gains were similar, but empty body-weight gain was significantly greater ($P < 0.05$) and carcass-weight gain non-significantly greater on the basal Hc diet.

A partition analysis of treatment sums of squares showed that the live-weight, empty body-weight and carcass-weight gains all gave very highly significant ($P < 0.001$) linear regressions. Consequently the gains were directly proportional to the level of acetate supplementation, as illustrated in Fig. 1.

Table 5. *Treatment means of gains in live weight, empty body-weight and carcass weight (kg) made during the 99 d experimental period by groups of six lambs receiving high-concentrate (Hc) or low-concentrate (Lc) diets with or without supplements of acetate*

Treatment	Live-weight gain	Empty body-weight gain	Carcass-weight gain
Hc unsupplemented	6·59	7·50	5·00
Hc + 228 kcal acetate/d	8·31	8·88	5·85
Hc + 456 kcal acetate/d	11·36	11·34	7·24
Hc + 684 kcal acetate/d	14·00	13·10	7·95
Lc unsupplemented	6·68	6·36	4·18
Lc + 228 kcal acetate/d	7·78	7·42	4·81
Lc + 456 kcal acetate/d	9·31	8·75	5·19
Lc + 684 kcal acetate/d	11·22	10·31	6·32
Standard error of difference between means (31 df)	0·47	0·48	0·41

A further partition analysis of treatment sums of squares was used to determine whether the linear regression relationships between weight gains and level of acetate supplement were different for each of the basal diets. For the relationship between live-weight gain and the level of acetate supplement, the linear regressions were of significantly different slope ($P < 0.001$) for the two basal diets. A difference was also apparent in terms of empty body-weight, although at a lower level of significance ($P < 0.01$).

The results relating carcass-weight gains to the supplemented level of acetate produced linear regressions with slopes which were different only at the $P < 0.08$ level of significance.

The linear regressions were used to compute the incremental response, over the experimental period of 99 d to a daily input of 100 kcal energy derived from acetate. These responses are shown in Table 6.

Carcass grades are not reported, but in general the heavier carcasses graded better. Consequently, of lambs receiving diet Hc, 66% graded A, while only 33% of lambs receiving diet Lc had grade A carcasses.

DISCUSSION

For empty body-weight and carcass-weight gains, 1847 kcal of metabolizable energy (ME) supplied by the Hc diet, which produced rumen liquor containing acetic, propionic and butyric acids in the molar proportions 64:21:12, was more

efficiently utilized than the same amount of ME supplied by diet Lc, which produced proportions of 74:14:10. This result confirms the findings of Blaxter & Wainman (1964) and Orskov & Allen (1966*c*). In terms of live-weight gain, the superiority of diet He was masked by the greater weight of gut contents in lambs receiving diet Lc.

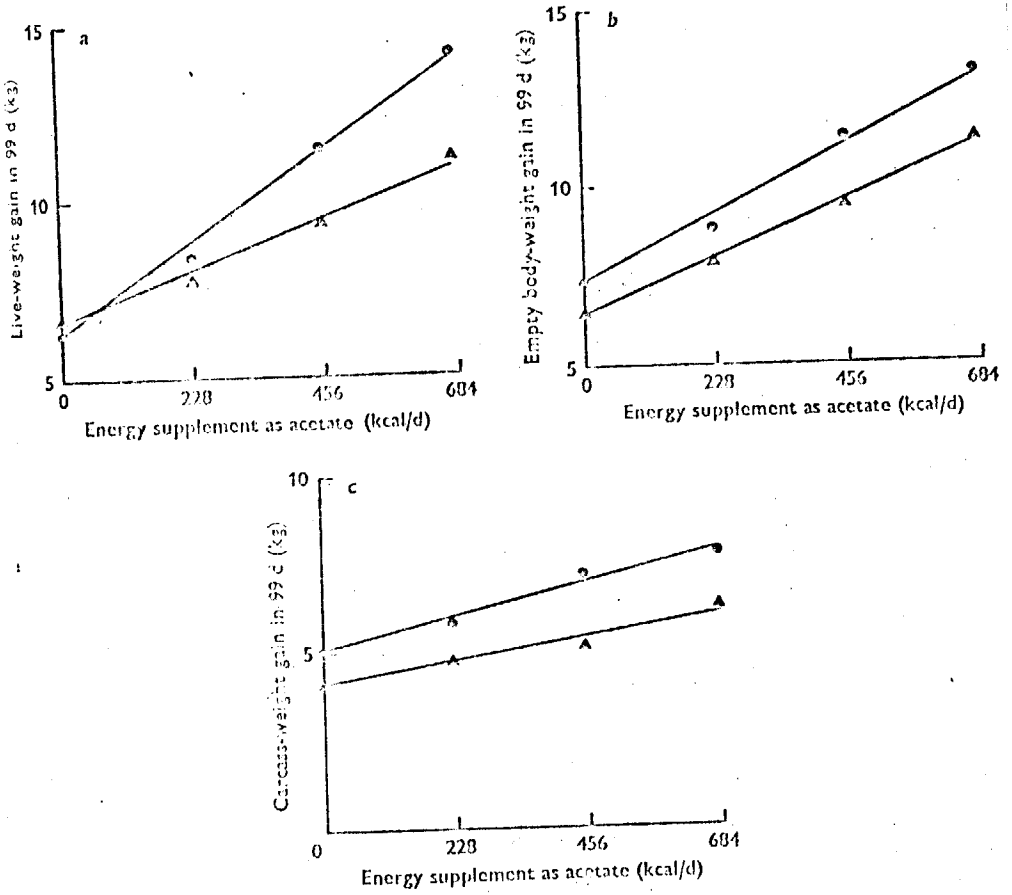

Fig. 1. (*a*) Live weight gains, (*b*) empty body-weight gains and (*c*) carcass-weight gains of lambs given high-concentrate (○) or low-concentrate (▲) diets, some of which were supplemented with acetate. Six lambs per treatment.

Table 6. *Calculated mean increments of live weight, empty body-weight and carcass weight (kg) in groups of eighteen lambs, following a supplement of* 100 *kcal/d of energy derived from acetate to a high-concentrate or low-concentrate diet over a* 99 *d experimental period*

Diet	Live weight	Empty body-weight	Carcass weight
High-concentrate	1·11	0·84	0·45*
Low-concentrate	0·67	0·58	0·30*

* These values were calculated using the regression lines of best fit, although in fact the slope of these lines did not differ significantly from each other ($P < 0{\cdot}08$).

The basal diets were given in amounts estimated to provide equal intakes of digestible organic matter, with ME intakes calculated on these amounts also being equal. However, this calculation assumes that ME is a constant proportion of digestible energy, which Blaxter & Wainman (1964) have demonstrated is not always the case. They showed that the net availability of ME for productive purposes was slightly increased when there was a high proportion of propionic acid in the rumen fermentation. In the experiment reported here, the basal He diet resulted in a rumen fermentation with a molar proportion of propionic acid of 21%, while the fermentation from the basal Lc diet produced only 14% propionic acid. A second factor is that, in order to achieve equal intakes of digestible organic matter with both diets, more of the basal Lc diet was given. This higher level of feeding can result in reduced availability of ME from the digestible organic matter (Agricultural Research Council, 1965). Both these factors tend to favour the He diet, although they would not be of sufficient magnitude to account for all the observed differences in performance. The remaining difference is probably accounted for by small changes in the efficiency of utilization of ME for maintenance, and larger changes for body gains, according to the dietary concentration of ME (Agricultural Research Council, 1965). The latter effect has been related to the type of rumen fermentation which a diet undergoes, and the utilization of the resultant end-products which are formed (Blaxter & Wainman, 1964).

This interpretation has been fully discussed by Orskov & Allen (1966*c*) who made a series of experiments in which isocaloric additions of salts of VFA to basal diets of hay and concentrate were utilized with similar efficiencies. They concluded that the lower efficiency of utilization of diets fermented in the rumen to produce high molar proportions of acetic acid might be due to energy losses occurring during the formation of acetic acid rather than during its subsequent utilization. Alternatively, the difference in the efficiency of utilization of high- and low-concentrate diets could be due to differences in the proportions of food digested in the forestomach and hindgut. Bull, Johnson & Reid (1967) have offered a further explanation, suggesting that the high heat increment of acetic acid recorded in short-term infusion experiments is not maintained over long periods, because metabolic mechanisms adapt to the increased load of acetic acid.

In the present experiment, larger amounts of acetate were given than in previous work, but there was still a linear response to graded supplements of acetate. Even with a daily supplement of 684 kcal energy, sufficient glucogenic material was probably available, since there was no sign of abnormal metabolism. The linearity of the regression extends to the unsupplemented basal diets, indicating a similar efficiency of utilization of ME derived from acetate, produced in the fermentation of the basal diet. It has been assumed that the acetate supplements had no effect on the fermentation of the basal diets (Orskov & Allen, 1966*a*), but the extent of interconversion of VFA in the rumen (Leng & Brett, 1966) which may have taken place is unknown. While Bergman, Reid, Murray, Brockway & Whitelaw (1965) have demonstrated that there is little direct interconversion between propionic and acetic acids, they did show that 61% of butyric acid carbon was in equilibrium with 20% of acetic acid carbon, and that 2–3 gram-atoms were interconverted each day.

The response of live-weight gain to acetate was due both to an increase in carcass weight and to increases in the weight of alimentary tract and its contents (Table 7). The increase in weight of alimentary tract contents was probably linked with the observed increase in water consumption of lambs given acetate. These effects help in the interpretation of the observed differences in efficiency of utilization of acetate when added to the different basal diets. For live-weight gain, acetate was more efficiently utilized when it supplemented the He diet ($P < 0.001$). This was largely because there were greater increases in weight of alimentary tract and its contents when the He diet was supplemented with acetate than when the Le diet was supplemented. For empty body-weight gain, acetate was again most efficiently utilized when supplementing the He diet, although at a lower level of statistical significance ($P < 0.05$). In this instance there was a greater proportionate increase in the weight of alimentary tract when the He diet was supplemented with acetate. With carcass-weight gain, the interaction between basal diet and acetate supplementation was not statistically significant, although there was a tendency towards greater gains in lambs fed acetate in conjunction with the He diet.

Table 7. *Mean weights of alimentary tract and its contents (kg) of six lambs per treatment*

Level of acetate supplementation (kcal/d)	0	228	456	684
High-concentrate diet: alimentary tract	3·28	3·44	3·76	4·17
contents	4·19	4·29	5·26	5·52
Low-concentrate diet: alimentary tract	3·36	3·43	3·48	3·76
contents	5·48	5·61	6·57	6·68

In this experiment there was no estimate of the tissue composition and calorific value of the body gains made by lambs on the different treatments. Elliot, Hogue, Myers & Loosli (1965) presented evidence which suggested that the carcass gains of lambs fed diets supplemented with isocaloric amounts of acetate or propionate were of similar composition. Since this evidence was based on carcass analysis of only two of the fourteen lambs receiving each treatment, it must be interpreted with some caution.

The results reported here are taken to indicate that the energy of acetic acid was utilized less efficiently when acetate was fed as a supplement to a diet which itself produced a high molar percentage of acetic acid in the rumen. This agrees with the tendency previously noted by Orskov & Allen (1966*c*) in an experiment in which high- and low-concentrate diets were supplemented with isocaloric quantities of acetate, propionate or butyrate. They found that whereas the utilization of acetate was better than that of propionate on the high-concentrate diet, it was worse than that of the other acids on the low-concentrate diet. They went on to suggest that their results, and those of Armstrong & Blaxter (1957) and Armstrong *et al.* (1958) could indicate that acetic acid concentration can be sufficiently high to cause wasteful oxidation. The ability of metabolic mechanisms to adapt to acetate absorption, as suggested by Bull *et al.* (1967), may have been exceeded in these experiments.

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THE INFLUENCE OF PRELIMINARY IRRITATION BY ACETIC ACID OR CROTON OIL ON SKIN TUMOUR PRODUCTION IN MICE AFTER A SINGLE APPLICATION OF DIMETHYLBENZANTHRACENE, BENZOPYRENE, OR DIBENZANTHRACENE

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PREVIOUS studies (Pound and Withers, 1963; Pound, 1963, 1966) have shown that a single treatment of the skin of mice by scarification or chemical means, a short interval before a standard tumour-initiating treatment with urethane, augmented the yield of skin tumours. The augmenting effect was confined to the area affected by the preliminary treatment. The view advanced was that proliferating cells are more susceptible to the tumour-initiating action of urethane, since, when the preliminary treatment was made with croton oil, the number of tumours produced could be related to the cellular proliferation that occurred in the skin. In particular, it correlated with the number of cells replicating DNA at the time of injection of the urethane (Pound, 1966, 1968). The simplest hypothesis to explain these findings is that urethane acts during the replication of DNA. This may also be the case with the carcinogenic hydrocarbons (Pound, 1968).

This paper records the results of experiments to test the effect of preliminary irritation on the number of tumours produced by 3 carcinogenic hydrocarbons in single dosage without the use of a promoting treatment.

MATERIALS AND METHODS

Mice

Random bred male mice of the strain "Hall" (Pound, 1962) from the Department of Pathology at the Royal Brisbane Hospital were used. The animals were about 7 weeks of age and weighed 24–26 g. at the beginning of the experiments. They were housed in stainless steel compartments, each holding 10 mice, with a bed of coarse sawdust that was changed weekly, and were fed the diet previously used (Pound and Withers, 1963) supplied, with water, in excess of their needs. The animal house was air-conditioned at 22° C.

Chemicals

Acetic acid, Osta Chemical Company, analytical reagent. Acetone, Ajax Products and Chemicals Pty. Ltd., analytical reagent. Croton oil, Stafford, Allen & Sons, London. 7,12 dimethylbenz(*a*)anthracene (DMBA); benzo(*a*)pyrene (BP); and dibenz(*a,h*)anthracene (DBA) were obtained from L. Light & Co., London, and were used without further purification.

EXPERIMENTAL

The hair of the skin of the back of the mice was clipped with electric clippers immediately before applications to the skin, care being taken to avoid injury since, in the light of previous work (Pound and Withers, 1963), this alone might influence the tumour yields. It was also clipped before counting tumours when necessary.

The hydrocarbons were applied to the whole area of the skin of the back by painting with approximately 0·25 ml. of solutions of the compounds in acetone 0·6%, 0·3%, 0·15% or 0·04% in the cases of BP and DMBA, and 0·3%, 0·15% or 0·04% in the case of DBA when the upper limit was defined by the solubility of the material. The area of skin covered was about 2·5 × 4·0 cm., that is about 10 sq. cm. The corresponding skin doses were therefore approximately 150, 75, 37, or 9 μg. per sq. cm. for BP and DMBA, and 75, 37, or 9 μg. per sq. cm. for DBA.

Experiments I, II and III

One thousand and eighty mice were distributed at random into compartments of 10. The compartments were arranged at random into 3 lots of 12 groups of 30 mice, i.e. 1 lot for each of the 3 hydrocarbons: Experiment I, DBA; Experiment II, BP; and Experiment III, DMBA.

For each experiment the mice were divided into 4 divisions, each comprising 3 groups of 30 mice. The mice in each division were given a preliminary application of 0·25 ml. of a 25% solution of acetic acid in acetone on the right side of the skin of the back, at 0, 24, 72 hours, or 9 days respectively before a single application of the carcinogenic hydrocarbon. The mice in the 3 groups of the 4 divisions were given an application of hydrocarbon to both sides of the skin of the back, 150, 37 or 9 μg. per sq. cm. for BP and DMBA and 75, 37 or 9 μg. per sq. cm. for DBA, respectively.

This gives a factorial arrangement in which the tumour yields on the 2 sides – treated and untreated with acetic acid—may be compared at 3 dose levels of the carcinogen and at 4 intervals of 0, 24, 72 hours and 9 days between the preliminary application of acetic acid and the application of the carcinogen. An interval of 0 hours between the 2 treatments means that the acetic acid solution was applied 15 minutes before the hydrocarbon, this being the time required for evaporation of the acetone solvent.

Experiment IV

Six hundred mice were divided at random into 3 lots of 200 and each lot divided into 5 groups of 40 mice.

The mice of 4 groups in each lot were given a preliminary application of 0·5 ml. of a 0·5% solution of croton oil in acetone to the whole area of the skin of the back at 0, 24, 72 hours and 9 days respectively, and the fifth group received no preliminary application, before the application of a carcinogenic hydrocarbon to the whole area of the skin of the back. The dose used was 75 μg. per sq. cm.

for all 3 hydrocarbons. One lot was treated with BP, the second lot with DMBA and the third lot with DBA.

Experiment V

Two control groups of 100 mice were painted once, one group with a 30% solution of acetic acid in acetone, the other group with 0·5% solution of croton oil in acetone. No carbinogenic hydrocarbons were administered.

The mice were examined at intervals for the presence of tumours in the treated area. A lesion was counted as a papilloma when it had reached a size of 1 mm. or more and persisted for 4 weeks or longer. A tumour was classified as malignant when it had grown progressively and had invaded the panniculus carnosus. Four sarcomata of the dermis developed in mice treated with the highest doses of DMBA but these are not included in the results. Malignant and doubtfully malignant growths were examined histologically but sections of clearly benign growths were not made. The number of tumours and their distribution on the skin of the back were recorded at fortnightly intervals, note being made of lesions that had regressed.

RESULTS

The application of croton oil or acetic acid in the amounts used leads promptly to acute inflammation in the skin. Epithelial hyperplasia soon begins and leads to keratin scaling from about the 4th or 5th day (Pound, 1968). In the control Experiment V, no tumours appeared during 40 weeks observation of the mice that had a single application of acetic acid (75 survivors) or croton oil (82 survivors). The natural incidence of skin papillomata in this strain of mouse at 12 months of age is less than one in a thousand over the whole area of the body (personal observation).

The smallest doses (9 μg. per sq. cm.) of the carcinogenic hydrocarbons did not produce any clinically obvious changes in the skin and did not alter the changes visible to the naked eye in areas previously treated with croton oil or acetic acid. The intermediate doses produced some epithelial scaling after about 4 or 5 days and was least with DBA and BP. It was most noticeable with DMBA when it was accompanied by some serous oozing in a few mice, mainly in areas that had been treated with acetic acid or croton oil. The largest doses of DBA and BP produced obvious keratin scaling but visible ulceration did not occur. The largest dose of DMBA (150 μg. per sq. cm.) produced severe changes in the skin submerging the effects of the preliminary treatments. There were often large areas of serous exudation which were slow in returning to normal, hair regrowth was delayed or in some cases did not occur, and the changes were accompanied by a constitutional disturbance.

The mice treated with the highest dose of DMBA did not gain as much in weight as those in the other groups. At the end of 30 weeks the animals were not in very good condition and, as the death rate appeared to be increasing, the experiments with DMBA were terminated at this time. Three of the mice treated with the highest dose of DMBA developed sarcomas associated with the thoracic cage and 1 of them developed a total of 6 papillomata well outside the treated area (which are not counted in the results). The occurrence of these tumours must be ascribed to absorption of the DMBA, which also may account for the deterioration in condition of these animals. In all the other groups the

mice remained in better condition and the death rates were not so great. These experiments were continued for 40 weeks.

In the mice of Experiment III treated with 150 μg. per sq. cm. of DMBA and a preliminary application of acetic acid at the same time and 1 day beforehand, a crop of small papillary growths occurred on the skin from the 8th week, a total of 7 on the side treated with acetic acid and 2 on the untreated side; these lesions regressed rapidly and do not appear in the results. This crop of transient lesions was not seen in Experiment IV in the mice given the preliminary application of croton oil before DMBA.

The time of appearance of each tumour counted, the tumours that regressed and those that became malignant, on each mouse, are set out in Tables I to IV. The mice that died with tumours are also shown.

The statistical analysis for Experiments I, II and III is based on the actual counts of tumours at each 10-week interval recorded in Table II; and for Experiment IV on the actual count at the termination of the experiment, Table IV.

Experiments I, II and III

Although the mice in these 3 experiments were randomized, the tumour yields were too small to allow the data to be treated as a whole and the experiments were considered individually.

Experiment I—DBA. The number of tumours was too small to estimate the effects of the various factors. At 40 weeks the total of 12 tumours on the right side treated with acetic acid, combining the results of the 3 dose levels, is significantly greater than the total of 3 tumours on the left untreated side ($\chi_1^2 = 5{\cdot}4$, $P < 0{\cdot}05$).

Experiment III—DMBA. Firstly, it was noted that the number of tumours on the right side is significantly greater than on the left side, after 10 weeks, 20 weeks and 30 weeks for dose 150 μg. per sq. cm. and after 20 weeks and 30 weeks for dose 37 μg. per sq. cm.

The tumour yields were treated as Poisson type counts, and analysis of variance performed after the square root transformation was applied.

The analysis showed that the differences between the right side and the left side, for the dose 150 μg. per sq. cm., were not related to the number of weeks of observation ($F_{2,6} = 0{\cdot}77$) but depended on the length of the interval between treatments ($F_{3,6} = 22{\cdot}51$, $P < 0{\cdot}01$). For dose 37 μg. per sq. cm. the difference between right and left sides again depended on the interval between treatments ($F_{3,3} = 92{\cdot}49$, $P < 0{\cdot}01$), but was not related to the period of observation ($F_{1,3} = 7{\cdot}67$, $P > 0{\cdot}5$).

Experiment II—BP.— A similar analysis was made to that for DMBA using the 20-, 30- and 40-week data for dose level 150 μg. per sq. cm. but only the 30- and 40-week data for dose 37 μg. per sq. cm. At 40 weeks the total yield of 22 tumours, combining the results of all dose levels, on the right side is significantly greater than the 9 tumours on the left side. Analysis of variance of the right *versus* left side differences at the 150 μg. per sq. cm. dose level gave no significant effect for weeks of observation ($F_{2,6} = 0{\cdot}87$) but a significant effect for interval between treatments ($F_{3,6} = 17{\cdot}37$, $P < 0{\cdot}01$). For the 37 μg. per sq. cm. dose level neither effect is significant (for intervals between treatments $F_{3,3} = 5{\cdot}20$, $P > 0{\cdot}1$), presumably because of the smallness of the yields.

Experiment IV

To consider the results of the individual carcinogens, in the mice treated with DMBA there are significant differences between the tumour yields ($\chi_1^2 = 14{\cdot}236$, $P = 0{\cdot}01$). The control group, given no preliminary treatment, differs significantly from the pretreated groups ($\chi_1^2 = 5{\cdot}074$, $P < 0{\cdot}05$) and there are significant differences between the pretreated groups ($\chi_3^2 = 9{\cdot}162$, $P < 0{\cdot}05$).

TABLE I. *Influence of a Preliminary Application of Acetic Acid at Intervals before Application of Hydrocarbon on Distribution and Time of Appearance of Tumours*

Carcinogen	Dose, µg. per sq. cm.	Interval between treatments	Survivors at 20 weeks	Distribution and time of appearance of tumours on each mouse (Left side/Right side)
DBA	75	0	28	34/0; 0/*21*; 0/*22*; 0/28, (*22*); 0/30
		1	26	36/0; 0/26; 0/32
		3	27	32/0; 0/32
		9	25	0/(20); 0/34
	37	0	27	0/22
		1	23	(22) 0; 0/32
		3	23	No tumours.
		9	28	0/24
	9	0	30	0/28
		1	29	No tumours.
		3	25	0/(24); (28)/0
		9	30	No tumours.
BP	150	0	26	0/36; 34/28, *16*; 0/16, *18*; 24/*16*, 24; 0/32
		1	27	28/0; 34/*24*; 0/18, 30; 0/26; 0/36
		3	27	(26), 28/*16*, *18*; 0/30
		9	27	28/0; 0/30
	37	0	23	28/30; 0/28, 34
		1	28	24/0; 0/26, 24
		3	30	No tumours.
		9	28	36/0
	9	0	25	No tumours.
		1	27	0/36
		3	27	No tumours.
		9	27	No tumours.
DMBA	150	0	23*	29/(12), 22, 26; 0/22; 0/*12*; 0/*12*; 0/*12*, *16*, (12); [0/18]; 12/*12*; 0/*16*, 16, 18, 24; 0/(14); 0/22; 18/(22); 0/20, (18); 0/*11*, *16*, 24; [0/*16*, *16*]; [14/16, 18]
		1	22	0/22; 0/10, 11; (10) (12), *12*, 14; 24/0; 24/(12); 0/10; 20/0; 22/(10); 0/(12), *16*; 0/22; [0/*16*, 22]
		3	22	22/0; 0/*16*, 19; 0/22, (*12*), (14); *12*, (14)/14; 0/22; 0/22, (10); 24/(22); 0/(18); 0/*16*
		9	28	(10)/0; 16/0; 14/10; 22/(10); [0/18]; *12*/0; (24)/0; 0/*12*
	37	0	25	0/14; 22/*12*, 18; 0/18; 0/16; 0/(16); 0/*12*; 22/0; 0/20, 22
		1	28	0/16; 0/18, 18, 18, 24; 0/24
		3	24	(16)/0
		9	26	0/24; 24/26; 22/0; 24/22, (24)
	9	0	28	0/22; 0/(18)
		1	28	0/*11*; 0/24
		3	28	No tumours.
		9	28	(12)/0; 22/(16).

Figures in parentheses are times of appearance of tumours that regressed.
Figures in italics are times of appearance of tumours that became malignant.
Figures in square brackets refer to mice that died with tumours.
* Tumours that appeared at ten weeks and regressed before twenty weeks are not shown.

TABLE II.—*The Influence of Preliminary Treatment with Acetic Acid on Number of Tumours Produced by Hydrocarbons*

DBA 75 µg. per sq. cm.
BP and DMBA
150 µg. per sq. cm.

Carcinogen	Interval between treatments	37 µg. per sq. cm.				9 µg. per sq. cm.			
		Surviving mice		Number of tumours		Surviving mice		Number of tumours	
		Number of mice	Left side	Right side		Number of mice	Left side	Right side	
DBA (40 weeks)	0	25	1	4		25	0	1	
	1	24	1	2		28	0	0	
	3	25	1	1		21	0	0	
	9	22	0	1		27	0	0	
DBA (30 weeks)	0	28	0	3		26	0	1	
	1	26	0	1		28	0	0	
	3	26	0	0		23	1	1	
	9	23	0	1*		28	0	0	
BP (40 weeks)	0	21	2	5		23	1	3	
	1	23	2	3		26	1	2	
	3	24	1	3		25	0	0	
	9	25	1	0		27	1	0	
BP (30 weeks)	0	21	1	6		23	1	2	
	1	23	1	4		28	1	2	
	3	25	2	3		30	0	0	
	9	25	1	1		27	0	0	
BP (20 weeks)	0	26	0	4		23	0	0	
	1	27	0	1		28	0	0	
	3	27	0	2		30	0	0	
	9	27	0	0		28	0	0	
DMBA (30 weeks)	0	19	3	17		24	2	8	
	1	17	4	6		23	1	6	
	3	16	3	2		24	0	0	
	9	22	4	2		26	3	3	
DMBA (20 weeks)	0	23	4	16		25	1	6	
	1	22	1	10		28	0	4	
	3	22	1	4		24	1	0	
	9	28	4	3		26	0	0	
DMBA (10 weeks)	0	23	2	22		27	0	0	
	1	26	2	10		30	0	0	
	3	28	4	9		26	0	0	
	9	29	1	2		29	0	0	

TABLE III.—*Influence of a Preliminary Application of Croton Oil at Various Intervals before Application of Hydrocarbons on the Yield of Tumours and Times of Appearance of Tumours*

Carcinogen	Dose, μg. per sq. cm.	Interval between treatments	Survivors of 20 weeks	Distribution and time of appearance of tumours on each mouse (Right side/Left side)
DBA (40 weeks)	75	0	33	34/0; 28 *20*, (32); 34/0; 28/0; (32)/0; [0/20]
		1	34	34/0; 28 *20*, (34); 0/22
		3	32	36 0; 22 0; 0 28
		9	37	*No tumours.*
		A	36	36 0; (24)/0; 0/32
BP (40 weeks)	75	0	34	24/0; 22/18; 18, 22/32, 32, (24); 0/36; [(22)/*16*, 22]
		1	34	36 0; 24 18; 32/*18*, 24; 28/24, 28; (24)/36; 0/24, 34
		3	34	(20)/0; *18*, 32 28; 26/24, 32; 0/24
		9	35	22, 28, 36 0; 0 30; 0/32
		A	39	28/0; 32 36, (22)
DMBA (30 weeks)	75	0	29	*12*, 18/0; 18 (22), 24; 24 0; 20/*11*, *18*, (18); (16), 22 16; 0/14; [0/*12*, 16]; [*10*, 18, 20/0]
		1	33	*12*, 16 18; *16*, *18*, (18), 24/12, 22; 16/20; 0 *18*, 22; 24/0; 16/*11*, 16, 24/20, 22; 18/0; 22, 26 0; [24/0]
		3	28	16, (18), (18)/20; 20, 22 0; 12/18, (22); 26/18, 24; 0 18, 22; 0 12, (20); (20)/24
		9	30	12, *10*; 22 0; 20 18, (18), (24), 24; (16) 16, 24
		A	34	12, 0; 20/*11*, (18); 16/22; 24/0; (24)/(24)

Figures in parentheses are times of appearance of tumours that regressed.
Figures in italics are times of appearance of tumours that became malignant.
Figures in square brackets refer to mice that died with tumours.
Forty mice in each group at beginning of experiment.
Groups A had no preliminary treatment with croton oil.

TABLE IV.—*The Influence of Preliminary Treatment with Croton Oil on Number of Tumours Produced by Hydrocarbons*

	Interval between treatments	Surviving mice: Number of mice	Surviving mice: Mice with tumours	Number of tumours
DBA, 75 μg./sq. cm. (40 weeks)	0	29	4	5
	1	29	3	4
	3	28	3	3
	9	31	0	0
	A	32	2	2
BP, 75 μg./sq. cm. (40 weeks)	0	31	4	8
	1	29	6	12
	3	27	3	5
	9	30	3	5
	A	34	2	3
DMBA, 75 μg./sq. cm. (30 weeks)	0	25	6	11
	1	27	9	22
	3	26	7	13
	9	28	4	8
	A	30	4	6

Groups A had *no* preliminary treatment. Forty mice in each group at beginning of experiment.

In the mice treated with BP no very significant differences appear (overall $\chi_4^2 = 8{\cdot}428$, $P > 0{\cdot}05$; difference between control and pretreated groups $\chi_1^2 = 3{\cdot}409$, $P > 0{\cdot}05$; differences between pretreated groups $\chi_3^2 = 5{\cdot}014$, $P > 0{\cdot}10$). Similarly no significant differences appear in the mice treated with DBA.

For an analysis of variance, if it is assumed that the difference due to the intervals between treatments can be estimated independently of the carcinogen used, the data can be treated as a 3×5 factorial (3 carcinogens *versus* 4 pretreatments and a control group). Tumour yields were treated as Poisson type counts.

Significant differences appear between the 3 carcinogens ($F_{2,8} = 21{\cdot}$, $P = 0{\cdot}001$). There are also significant differences between the control group and the pretreatment groups ($F_{1,8} = 7{\cdot}414$, $P < 0{\cdot}05$). In addition, significant differences exist between the 4 pretreatment groups ($F_{3,8} = 6{\cdot}489$, $P < 0{\cdot}0$). Comparing the pretreated groups with the controls the differences at the 0 interval and the 1 day interval are significant, $P < 0{\cdot}05$, and $P = 0{\cdot}01$ respectively. The difference between the controls and the 3 day interval is of doubtful significance and the remaining difference not significant at all.

In summary, there is clear evidence that, when one side of the skin of the back of mice is treated with acetic acid before application of any of the 3 hydrocarbons to the whole area of the skin of the back, the number of tumours produced is greater on the pretreated side. In the case of DMBA at the 150 μg. per sq. cm. and 37 μg. per sq. cm. dose levels and in the case of BP at the 150 μg. per sq. cm. dose level the increase varied with the interval between the 2 treatments and was independent of the period of observation. The tumour yields were increased when there was no significant interval between the 2 treatments and at an interval of 24 hours. A similar trend appears at the lower dose levels of DMBA and BP and also when DBA was the carcinogen although the tumour yields were too small for statistical treatment.

Similarly when mice were given a preliminary treatment with croton oil before the application of the carcinogens the tumour yield was increased when the interval between treatments was zero and 24 hours. A doubtful effect was found at an interval of 3 days. The variation with the interval between treatments is significant only with DMBA, but a similar trend is seen in the results with DBA and BP.

From inspection of Tables I and III it is apparent that these trends are maintained in mice that died with tumours. Also, the number of tumours that regressed was greater on skin that had the preliminary treatment.

It is to be noted that the tumour yields in areas of skin that did not have the preliminary treatment with acetic acid (in Experiments I, II, and III), appear to be similar, allowing for variation in dosage levels, to the tumour yields in mice that had no pretreatment with croton oil in Experiment IV. Similarly, in areas of skin subjected to preliminary treatment with acetic acid in Experiments I, II and III the increase in tumour yields is of similar order to the increase in the tumour yields in similar areas of mice that had a preliminary treatment with croton oil, again allowing for the variation in dosage levels.

It will be observed from Tables I and III that tumours appear earlier on the average with the more potent carcinogens. The tumour yields are not such as to treat this statistically.

Lastly, it is apparent from Tables I and III that malignant tumours appear

to be more frequent in areas given the preliminary treatment on the one hand and with the more potent carcinogens on the other. However, the ratio of malignant to benign tumours does not vary significantly between the different areas. The frequency of malignant tumours therefore appears to be related to the number of tumours in any particular area, and perhaps also to the length of time tumours have been under observation.

DISCUSSION

The production of skin tumours in mice by a single application of a carcinogenic hydrocarbon has been reported, for example, using 3 : 4-benzopyrene (Bielschowsky and Bullough, 1949), 20-methylcholanthrene (Mider and Morton, 1939; Cramer and Stowell, 1943) and 9 : 10 dimethyl 1 : 2-benzanthracene (Law, 1941; Cramer and Stowell, 1943) and 9 : 10 dimethyl 1 : 2-benzanthracene (Andreasen and Engelbreth-Holm, 1953; Borum, 1954; Terracini, Shubik and Andreasen and Engelbreth-Holm, 1953; Borum, 1954; Terracini, Shubik and Della Porta, 1960). The tumours appeared in small numbers after a long latent period; most have been benign but some were malignant. Allowing for the differing strains of mice used by different workers and for the uncertainties of dosage per unit area of skin, DMBA appears to be the most active as judged by the numbers of tumours produced.

The number of tumours to be expected in the experiments reported in this paper is therefore likely to be small, as was found, although a quantitative comparison of the results with those of other authors is invalid because of the differing strains of mice, uncertainty in the dosage per unit area used by other authors, the particular sample of DMBA, DBA and BP used and possibly other factors. Dose response data have been reported with DMBA (Terracini *et al.*, 1960) and the present results follow a similar pattern. Nonetheless, it is clear that DMBA produced more tumours than DBA or BP, and that BP was more active than DBA, at least with the samples used.

A preliminary application of acetic acid or croton oil at the same time as, or a short interval before, the application of any of the 3 hydrocarbons increased the number of tumours produced. The relative increase when the treated side of mice that had the preliminary treatment of acetic acid on one side of the skin of the back was compared with the untreated side was similar to the relative increase when mice that had a preliminary treatment with croton oil to the whole of the back were compared with mice that had no preliminary treatment. The augmenting effect is therefore localized to the area affected by the preliminary treatment and not the result of general metabolic changes; although formal demonstration of this would depend on the results of similar experiments in which the manner of the preliminary treatments were reversed. However, since the tumour yields in untreated areas of skin in the 2 experiments were similar, these experiments would appear to be redundant. The amounts of acetic acid and croton oil applied were the highest that could be used without producing chemical ulceration of the skin and led to similar degrees of epithelial scaling as judged by the naked eye.

Croton oil is a potent promoting agent for the production of skin tumours in mice subjected to the action of an initiating agent such as urethane (Salaman and Roe, 1953) or the carcinogenic hydrocarbons (Mottram, 1944*a*; Berenblum and Shubik, 1947); and on repeated application alone appears to have a minor carcinogenic effect (Roe, 1956; Boutwell, Bosch and Rusch, 1957). However, acetic acid has no promoting activity when carcinogenic hydrocarbons are employed as initiating agents and does not itself lead to the production of tumours (Gwynn and Salaman, 1953). The tumour augmenting effect is therefore, as in the case of the similar augmenting effect of preliminary irritation on urethane carcinogenesis (Pound and Withers, 1963; Pound, 1966), not related to these properties but is probably related to the common property of producing inflammation and cell proliferation in the skin.

However, in the case of experiments in which hydrocarbons are applied to the skin, other factors merit consideration. Thus, the number of tumours produced after a single application of DMBA appears to be related to the stage of the hair cycle at the time, more tumours being produced if this is in the resting phase (Andreasen and Engelbreth-Holm, 1953; Borum, 1954). From study of sections of mice of the same age and weight, the hair cycle of about one third of the mice used in the present experiments is in the late catagen phase and of the remainder in the resting phase. The possibility that the results can be due to a variation from this source is therefore unlikely and can be excluded since treated sides were compared with the untreated sides of the same animals in which the cycle would be in the same phase on either side. However, it has been shown that the hair cycle effect is explained mainly by retention of the hydrocarbon in the resting hair follicles (Berenblum, Haran-Ghera and Trainin, 1958).

After the application of croton oil in the amount used, vascular dilatation and oedema develop rapidly and increase to about the 12th hour, after which the changes regress slowly to approach normal after about 24 hours. These changes are accompanied by a leucocytic infiltration which, however, persists for longer than 24 hours. The number of nuclei of the epidermis labelled in radio autographs of sections of the skin, when the mice were injected with 10 μc tritiated thymidine 30 minutes before being killed, increased very rapidly from the 9th hour to a maximum at the 18th hour and then receded to normal at about the 9th day, whereas mitotic counts in the epidermis increased slowly from the 15th hour to a maximum at about 36 hours and then receded slowly to normal at about the 9th day (Pound, 1968). A similar pattern of these events was reported by Iversen and Evensen (1962). Forty-eight hours after the application of croton oil the epidermis is increased in thickness and soon begins to differentiate a layer of keratin. The epidermal changes extend into the superficial part of the hair follicles. Although no study has been made of the changes following an application of acetic acid, the changes visible to the naked eye are similar and the microscopic events are likely to be substantially the same.

It seems possible that the proliferating epidermis, on the surface and in the superficial part of the hair follicles, might have an increased capacity to retain hydrocarbons applied to the skin but if this factor contributed significantly to the results, the tumour yield would be expected to be augmented when the hydrocarbon was applied on the 3rd and 9th days after the preliminary application. This was not the case.

In previous studies concerning the augmenting effect of irritants on tumour initiation by urethane (Pound, 1968) it has been shown that the increased tumour yield correlates with cellular events in the skin, in particular with the number of cells replicating DNA, at the time of injection of the urethane. This suggests that this chemical exerts its tumour initiating action during this phase.

SUMMARY

Groups of mice were given a single application of acetic acid to one side of the skin of the back. Other groups were given an application of croton oil to the whole area of the skin of the back, a control group had no application of croton oil. At the same time as, 24 hours, 72 hours, or 9 days after the application of acetic acid or croton oil, the mice were given a single application of one of the carcinogenic hydrocarbons DMBA, BP or DBA, to the whole area of the skin of the back.

The number of tumours produced was greater in areas that had the preliminary treatment with acetic acid or croton oil at the same time or 24 hours before the carcinogen. There was a doubtful effect at an interval of 3 days and no effect at 9 days.

It is considered that proliferating epidermal cells are more susceptible to the action of the carcinogens perhaps during replication of DNA.

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It seems possible that the carcinogenic hydrocarbons also act during a similar period of the cell cycle. The demonstration of this in experiments in which the hydrocarbon is applied to the skin is likely to be clouded by the facts that, on the one hand, these compounds persist in the tissue for a considerable time, and, on the other hand, they themselves lead to epithelial hyperplasia in the skin (Orr, 1938). The sharp increase in tumour yields found when urethane was injected as an initiating agent at various times after a preliminary irritating treatment of the skin could not be expected. This indeed was the reason for selection of the particular intervals between the preliminary treatment and the application of the hydrocarbon used in the present experiments.

Many years ago Mottram (1944*b*) reported that the number of tumours produced by benzopyrene (used as an initiating agent followed by repeated applications of croton oil which he referred to as a developing factor, but which is now referred to as a promoting agent) was increased if the skin was given a preliminary treatment with croton oil. He showed that the preliminary treatment resulted in a great increase in the mitotic counts in the skin. Similarly, he found that a preliminary treatment with cantharidin under conditions that depressed the mitotic counts decreased the tumour yields, and under conditions that increased the mitotic counts cantharidin increased the tumour yields. Mottram (1945) also found that more tumours were produced if the benzopyrene was applied at midnight rather than at midday which he ascribed to higher mitotic rates at this time, a view that has not been supported by subsequent results (Bielschowsky and Bullough, 1949). He concluded that "the genesis of tumours represents action on cell division". In spite of the revolutionary nature of this concept, for various reasons experiments that would confirm or refute this thesis have only been performed recently.

Frei and Ritchie (1964) have confirmed that DMBA, followed by promoting treatment with croton oil, produces more tumours if applied at midnight than if applied at midday; and Shinozuka and Ritchie (1967) have reported that a preliminary application of croton oil 23 hours before the application of DMBA, as an initiating agent, augmented the yield of tumours. These results were interpreted as compatible with the theory that the carcinogens act on cells synthesizing DNA.

A similar result was reported (Pound, 1968) using DBA as an initiating agent : the tumour yield was increased at intervals of 0, 24 hours and 3 days, and : at 9 days, between a preliminary application of acetic acid and the carcinogen. most identical results have been obtained (Pound, to be published) when BP : DMBA were the initiating agents. While these results completely dissociate augmenting effect of the preliminary treatment from promoting activity, y do not give the clear correlation with the pattern of DNA synthesis found n urethane. Nor should such a pattern be expected for reasons noted above. further experiments (Pound, 1968) it was reported that partial hepatectomy fore injection of urethane or oral administration of DMBA increased the number tumours produced in the liver, an effect that may be related to the active generation of the liver during the presence of the carcinogen.

It seems possible therefore that tissues induced to proliferate rapidly become more susceptible to the action of a carcinogen, as a general phenomenon. The evidence that possibly identifies the sensitive phase in cell life as the phase of replication of DNA comes mainly from the work with urethane (Pound, 1968).

VEGETABLE FLAVORS

Flash Exchange Gas Chromatography for the Analysis of Potential Flavor Components of Peas

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The technique of flash exchange gas chromatography was applied to the determination of aldehydes, ketones, and acids in the steam distillate of peas. The compounds identified were acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, biacetyl, formic acid, acetic acid, and isovaleric acid. All these components occurred at a concentration of less than 3 p.p.m. on a fresh-weight basis. A mixture of these compounds at the concentration levels found did not reproduce the characteristic odor of pea steam distillate.

The long-range goal of flavor research is to improve flavor so that abundant, nutritious foods will be consumed in increased use. It is most desirable that control techniques be developed which could be applied to small, specific quantities of material. Accuracy need not be greater than that required for a starting point in taste panel evaluation. With this type of research tool, studies of plant breeding, crop selection, harvesting practice, processing, and storage could be made with improved flavor as an objective.

A useful technique for the determination of carbonyl compounds and acids was developed which required only a few milligrams of compounds (3). Aldehydes, ketones, and acids are isolated as solid derivatives, regenerated in rapid exchange reactions, and volatilized

Table I. 2,4-DNPH Prepared from Steam Distillate of Peas

Lot No.	2,4-DNPH, Mg. 1958[a]	1959[b]
1	25.4	11.7
2	21.2	11.5
3	23.6	8.9
4	15.1	11.3

[a] Insoluble 2,4-DNPH collected by filtration.
[b] Extracted. Filtration gave 0.7 to 2.8 mg. of 2,4-DNPH.

Table II. Concentration of Volatile Carbonyl Compounds in Initial Steam Distillate of Peas

Compound	Av. Concn., Mg./Kg. Fresh Wt. 1958	1959
Acetaldehyde	2.4	0.56
Acetone	0.35	0.12
Propionaldehyde	0.17	0.00
n-Butyraldehyde	0.15	0.06
n-Valeraldehyde	0.60	0.44

Table III. Change in Volatile Carbonyl Composition of Steam Distillate of Peas (1958) with Time

Time, Min.	Volume Distillate, Ml.	Weight 2,4-DNPH, Mg.	Weight Due to Biacetyl	Carbonyl Content, Mg./Kg., Fresh Wt.	
				Biacetyl	0.61
				Acetaldehyde	0.62 (0.70)
				Acetone	0.14 (0.52)
				Propionaldehyde	0.046 (0.10)
				n-Butyraldehyde	0.055 (0.11)
33	275	15.1	6.27		
57	263	5.05	4.04	Biacetyl	0.39
81	310	3.59	3.58	Biacetyl	0.34
99	258	1.96	3.02	Biacetyl	0.29

directly into a gas chromatography unit for separation and identification. Aldehydes and ketones are determined by regeneration from 2,4-dinitrophenylhydrazones (2,4-DNPH) with α-ketoglutaric acid. Monobasic, carboxylic acids are transformed from their potassium salts into ethyl esters by heating with potassium ethylsulfate.

Experimental

The peas used were portions of large lots of blanched, frozen, Perfection (commercial) peas prepared on June 25, 1958, and June 19, 1959, at Milton, Ont. The only difference in the two lots was the earlier maturity and longer blanching time for the 1959 lot. The peas (2 kg. samples) were thawed for 4 hours, ground with 1000 ml. of distilled water in a food blender, and steam distilled at atmospheric pressure with a boiling water bath as a heat source. A heating period of 1 hour was necessary to reach distillation temperature. A first distillate of 250 ml. was collected by passing in clean steam for about a half hour. A gas trap containing 2,4-dinitrophenylhydrazine reagent was used to ensure that all volatile carbonyl compounds were trapped. The distillate was treated with a solution of 200 mg. of 2,4-dinitrophenylhydrazine in 20 ml. of concentrated hydrochloric acid. The turbid suspension was heated to boiling for 5 minutes, cooled slowly to room temperature, and stored overnight in a refrigerator. The 2,4-DNPH was collected by filtration, washed with 2*N* hydrochloric acid, then water, and dried to constant weight at reduced pressure over phosphorus pentoxide. The volatile C_1 to C_5 aldehydes and ketones in the mixed 2,4-DNPH were determined by the flash exchange reaction with excess α-ketoglutaric acid (*5*).

After the predominant derivatives had been removed by filtration, the filtrate and washings were diluted to 450 ml. and extracted continuously with purified petroleum ether for 16 hours. The yellow-colored extract, 250 ml., was washed with three 50-ml. portions of water, dried over anhydrous sodium sulfate, and evaporated.

For the determination of acids, the steam distillate was treated with 0.1*N* potassium hydroxide to pH 8.5 and evaporated. The resulting mixture of potassium salts was dried over phosphorus pentoxide at room temperature and pressure. The volatile C_1 to C_5 monobasic, carboxylic acids were determined by flash exchange with potassium ethylsulfate.

Results and Discussion

Carbonyl compounds are present in the steam distillate of peas at a very low concentration. Indeed, if blanched, frozen, intact peas are heated with water, there is almost no detectable carbonyl compound in the distillate. It was necessary to steam distill the pulverized peas to get sufficient carbonyl derivative for analysis. The results of several such steam distillations are shown in Table I. These results are in marked contrast to those reported by Silberstein (*3*) and Wager (*6*). Silberstein obtained 50 to 300 mg. of 2,4-DNPH in 2 kg. of peas, by steam distillation at pressure, which may account for part of the difference in results. The discrepancy is due in part to such factors as variety of pea, maturity at harvest, type of blanching used, and storage conditions. Silberstein demonstrated that blanched peas had a much lower volatile carbonyl content than fresh peas.

The results of the analyses of the mixed 2,4-DNPH from pea distillates are shown in Table II. The predominant carbonyl compounds are acetaldehyde, acetone, propionaldehyde, *n*-butyraldehyde, *n*-valeraldehyde, and biacetyl. The latter compound was determined separately by colorimetric methods and the results are shown in Table III. The difference in amount and distribution of the carbonyl compounds in the 1958 and 1959 pea lots is interesting. The 1959 material had a much lower volatile carbonyl content and was different from the 1958 material by the absence of propionaldehyde and the presence of *n*-valeraldehyde.

A brief study of the variation of carbonyl content with time of distillation was made and the data are shown in Table III. It was found that after the first distillation fraction, the major part of the weight of the precipitated 2,4-DNPH was due to biacetyl bis-(2,4-dinitrophenylhydrazine). The concentration of the biacetyl appears to decrease to a limiting value with time. A separate study (*1*) of acetoin and biacetyl in peas showed that acetoin is the predominant material with a concentration of 260 mg. per kg. of fresh weight. The acetoin is not volatile with steam under the conditions of the distillation.

Extraction of the filtrate after collection of the precipitated 2,4-DNPH produced considerable material (11.7 mg. from the first fraction of 1958 run No. 4). Analysis of the extracted material is shown within parentheses in Table III. It was expected that some trace constituents would appear in the extracted material. This did not occur, because only the four primary carbonyl compounds were found at both the 75° and the 150° C. column temperatures.

Volatile acids in the steam distillate

Table IV. Volatile Acids in Steam Distillate of Peas (1959)

Acid	Concn., Mg./Kg. Fresh Wt. Run No. 1	2	Av.
Formic	0.33	0.46	0.39
Acetic	0.024	0.029	0.027
Isovaleric	0.067	0.060	0.064

1958 peas are shown in Table IV. ...d composition is relatively simple, ...ric acid is the primary constituent ... small amounts of acetic and iso- ... acids also present. Formic acid ... been detected previously in canned ...s (3).

The steam distillate of peas has the ... characteristic odor of cooked ... If the components responsible ... the odor can be identified, a sub- ... part of pea flavor would be ...stroyed. Earlier work in this field ... indicated that carbonyl compounds are an important part of pea odor (1). Mixtures of the carbonyl compounds identified in this study do not approach the cooked pea odor. Work on the isolation and identification of the odorous components of pea steam distillate is being continued.

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PRESERVATION OF WET LEAF-PROTEIN CONCENTRATES

By M. S. SUBBA RAO, NARENDRA SINGH and G. PRASANNAPPA

Simple methods of preservation of wet leaf-protein cakes for food were studied. The use of a pickle preservative emulsion gave encouraging results, and 2% residual acetic acid in wet cakes preserved them satisfactorily. The initial microbial load could be reduced by lowering the pH with either HCl or acetic acid, but further microbial growth could be checked only with acetic acid. Dilute acetic acid of 4% or 10% strength could be mixed in 1 : 1 or 1 : 4 w/v proportions, respectively, with the leaf-protein slurry, before pressing, to get the desired residual acid in wet protein cakes. An even dispersion of acid between the cake and exudate during pressing made a successive re-use of the latter possible, with less acetic acid added to it than would otherwise have been needed.

Introduction

The leaf proteins have been accepted as promising materials for increasing food protein supplies to meet the growing demands in the world.[1] As a result of systematic work for over 25 years, the technology has been successfully developed to make bulk production of edible leaf proteins possible.[2-4] The leaf-protein concentrates could be used either as wet cake, fresh from the press, or as a dried powder. The use of developed techniques of drying is within the scope of investment and skill available for production of leaf proteins on an industrial scale, but such facilities are limited when production is for small communities. This is particularly true in villages in the less industrialised countries, where the use of wet protein cake would be the more suitable choice. In addition, the drying gives rise to a grittiness in the final product which is not acceptable in foods, and air-drying may also reduce the

nutritive value of leaf proteins.[5] Hence, wet leaf-protein cake is favoured, but is is liable to undergo microbial spoilage unless stored under frozen conditions.

The use of common salt in the wet cake at 12% concentration, can prevent microbial spoilage,* but limits the scope of utilisation of such materials in food preparations. Attempts to use a pickle preservative emulsion containing acetic acid and orange peel oil gave encouraging results.[6] Acetic acid alone could also be used as a preservative, and the use of these preservatives for wet leaf-protein cakes was considered a useful topic for investigation.

Materials and methods

The leaf extracts were made from the green vegetation of lucerne (*Medicago sativa*) using a batch extractor.[4] The proteins were coagulated by injecting steam into the extract, and the treated extract was altered through drill stockings and given two washes with water. The leaf-protein slurry retained in the stockings was kept overnight in a beam press to form a wet leaf-protein cake (LPC), usually containing 65–75% moisture. Details of the formulations of the pickle preservative emulsion (PPE) are given in Table I.

The microbial counts for total viable cells, spores and mould and yeast cells in test materials were made using the A.P.H.A. method,[7] with incubation at 32 for 48 h. The results are expressed on a moisture-free basis, unless it is stated otherwise. Moisture was determined by drying a known weight of test material to a constant weight at 80 85 . Acidity was determined according to the A.O.A.C. method. All materials were maintained at room temperature for storage studies.

Results and Discussion

The preliminary studies on preservation of wet LPC were mainly concerned with visible microbial growth and apparent off-flavour during storage. The results with various formulations of the pickle preservative emulsion and with vinegar are presented in Table I. From consideration of the acetic acid levels in treatments which had shown no spoilage over several months, one with PPE at 1 : 1 w/w and the other with vinegar at 2 : 1 w/v, the inference was that about 2% residual acid in the wet cake was adequate to preserve it. Since the moisture content of wet LPC might vary from batch to batch, studies were done with LPC with moisture variations of between 70 and 90%. Again about 2% residual acetic acid preserved the material satisfactorily. Based on these preliminary observations, the following studies were carried out.

TABLE I

Preservation of wet leaf-protein cakes with pickle preservative emulsion and vinegar

(Wet leaf-protein cake was minced in the specified w/w ratio with various formulations of pickle preservative emulsion or the w/v ratio with vinegar shelf life was assessed from visible microbial growth)

Formulation	Ratio (w/w)	Shelf life
Standard (contained 5% acetic acid and 0·2% orange peel oil)	1 : 1	>18 months
Standard (contained 5% acetic acid and 0·2% orange peel oil)	9 : 1	<9 days
Standard (contained 5% acetic acid and 0·2% orange peel oil)	4 : 1	<8, <9, >42 days
A (contained 10% acetic acid and 0·2% orange peel oil)	4 : 1	>77 days
B (contained 8·6% acetic acid and 0·33% orange peel oil)	4 : 1	>77 days
C (contained 5% acetic acid and 0·4% orange peel oil)	4 : 1	>77 days
D (contained 4·4% acetic acid and 0·7% orange peel oil)	4 : 1	>77 days
Vinegar (6% acetic acid)	4 : 1 (w/v)	<10 days
Vinegar (6% acetic acid)	2 : 1 (w/v)	>15 months

The leaf extract, leaf-protein slurry and wet LPC obtained from the slurry in 2 batches were analysed for initial microbial load. The wet LPC from a part of the slurry from one batch (I) treated with HCl to lower the pH, and that from the other batch (II) treated with 10% acetic acid (4 : 1 w/v) were also similarly analysed. The results are presented in Table II. There were batch variations, but the LPS in both batches had a considerably lower microbial load than the leaf extracts, on account of the steam treatment. In the pressed untreated cake there was a build-up of microbial load because of favourable conditions of pH and moisture. The treatments with either HCl or acetic acid decreased the initial

* Pirie, N. W., Private communication

TABLE II

Initial microbial load at different stages of preparation of leaf-protein cake

	pH		Acidity* (%)		Microbial count per g of dry material: Total viable cells (10^3)		Spores (10^3)		Mould and yeast (10^3)	
	I	II	I	II	I	II	I	II	I	II
Leaf extract	6·5	6·4	0·2	0·18	736	6668	576	23190	91	145
Leaf-protein slurry	6·9	6·8	0·1	0·05	36	88	220	19300	6	1
LPC	6·7	7·0	0·1	0·1	102	39490	829	∞**	11	9
LPC treated with HCl	4·5	—	0·1	—	3	—	52	—	8	—
LPC treated with acetic acid	—	4·0	—	2·1	—	6	—	116	—	1

* Acidity is expressed as HCl in all treatments except the LPC treated with acetic acid where it is expressed as acetic acid
** Number too high to count

microbial load in the LPC mainly because of lowering of the pH, which is inhibitory to most of the normal microbial contaminants.

The initial microbial contaminants on different cakes were identified broadly as follows. The heavily contaminated LPC from untreated slurry contained predominantly gram-positive spore-forming rods with a few gram-negative rods and mould and yeast cells, while the LPC from slurry treated with HCl or acetic acid showed only a few gram-positive spore-forming rods.

As both HCl and acetic acid reduced the initial mocrobial load, the storage behaviour of wet cakes from HCl and/or acetic acid treatments was studied for a period of 16 days and the results are presented in Table III. The single acid treatments did not show appreciable differences in pH, while the double acid treatment resulted in a lower pH. The initial microbial load in LPC–HCl was appreciably higher than that in the other two and there was also rapid microbial growth, the entire mass becoming covered with moulds and yeasts within 4 days. On the other hand, treatment with acetic acid not only reduced the initial microbial load but also prevented the growth of micro-organisms during storage.

A few large batches of leaf-protein slurry mixed 4 : 1 and 1 : 1 w/v with 10% and 4% acetic acid, respectively, gave 2% residual acid in the pressed LPC, and showed satisfactory storage behaviour.

Since the acid was partitioned in equal proportions between the pressed cake and the exudate, and the latter indicated no microbial build-up under storage for 16 days, the possibility of re-using exudate was investigated. A 16-day-old exudate with its acetic acid content adjusted to 4% was re-used for treating a fresh lot of leaf-protein slurry, and the storage behaviour of the wet LPC and re-used exudate from this treatment was studied (Table IV). Neither of them showed any appreciable change in microbial load during storage for up to 16 days.

TABLE III

Storage behaviour of leaf-protein cakes treated with HCl and/or acetic acid

(Leaf protein cakes were prepared from leaf protein slurry treated with HCl to pH below 4·0 (LPC-HCl), from slurry treated with acetic acid to give about 2% residual acid (LPC-acetic acid) and from slurry given both treatments (LPC-HCl acetic acid))

	Storage (days)	Total viable cells (10^5)	Spores (10^1)	Mould and yeast (10^3)
LPC-HCl (pH 3·65–3·7; acidity 0·12% as HCl)	0	9·24	47470	0·34**
	2	1559	49780	∝
	4*	—	—	—
	16*	—	—	—
LPC-Ho[illegible] acetic acid (pH 3·2–3·25; acidity 1·9% as acetic acid)	0	1·2	141	Nil
	2	0·9	236	,,
	4	0·8	239	,,
	16	0·43	191	,,
LPC-[illegible] acid (pH 3·8–3·85; acidity 0·8% as acetic acid)	0	0·5	165	,,
	2	0·3	108	,,
	4	0·22	109	,,
	16	0·11	109	,,

* Within 4 days the cake was covered with mould and yeast which made any subsequent count impossible

** Too numerous to count

TABLE IV

Re-use of exudate

(The acidity of a 16-day-old exudate, containing 1·99% acetic acid, was brought up to 4% with added acetic acid. This exudate was mixed with leaf-protein slurry, 1 : 1 w/v, and the LPC and re-used exudate, were analysed for microbial load. Both samples showed acidity 1·85–2·00% as acetic acid pH 3·6–3·7)

	Storage (days)	Microbial count: Total viable cells (10^5)	Spores (10^1)	Mould and yeast (10^3)
Exudate*	0	0·1	2	Nil
	4	0·1	2	,,
	8	0·2	2	,,
	16	0·2	3	,,
LPC	0	4·9	68·8	,,
	4	0·4	4·5	,,
	8	0·8	24·3	,,
	16	1·2	32·1	,,

* Microbial count expressed per ml of exudate

TABLE V

Successive re-use of exudate

(Exudate from one batch, with acetic acid added to bring its level to 4%, was used for treating the protein coagulum slurry from 4 batches, successfully (A → B → C → D). Leaf-protein cakes from such batches were analysed for initial and final microbial loads. Acidity 1·8–2·1% as acetic acid; pH 3·9–4·0)

	Storage (days)	Microbial count: Total viable cells (10^5)	Spores (10^1)	Mould and yeast (10^3)
LPC–A	0	0·1	49·5	Nil
	23	0·05	99·0	,,
LPC–B	0	0·04	44·1	,,
	20	0·04	87·7	,,
LPC–C	0	0·05	134·5	,,
	17	0·01	134·0	,,
LPC–D	0	0·09	134·0	,,
	11	0·05	134·0	,,

With this evidence the possibility of successive re-use of exudate was tested. The exudate from each batch was used for another batch in 4 successive batches on 4 separate days. The initial microbial counts and those after different periods of storage are shown in Table V. In spite of batch variations there were no appreciable differences in initial microbial loads in different LPC samples. The final microbial count in stored LPC also did not appreciably differ from the initial load in any of the batches. These results indicated that exudate could be successively re-used from batch to batch after fortification with acetic acid to the desired level. This would economise the use of acetic acid and thereby bring down the cost of preservation of wet protein cakes.

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No. 48 - The Separation and Determination of Carboxylic Acids from C_1 to C_6 by Partition Chromatography on a Silica Column, I. General Study

by Roger Raveux and Josy Bové

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The various factors which interfere in chromatography on a silica column of carboxylic acids from C_1 to C_6 were studied.

The quality of the silica is the most important point to consider. Lots of silica from the same province and the same commercial origin may show sometimes considerable differences relating to adsorbant qualities. Silica which has adsorbant properties is not usable, and it is often difficult, if not impossible to eliminate these unfortunate properties. When one has several lots of commercial silica differing by their numbers of manufacture, it is important to effect with each one a control separation in order to identify the adsorbant silica and to reject it. For example, silica of Mallinckrodt lots XNB-1 and YDR was excellent, that of ACM was poor.

Column chromatography has, in comparison to paper chromatography, the advantage of allowing the separation and determination of large quantities of a substance by very small quantities of another compound. In the separation and determination of organic acids by column chromatography, three main processes are evident: chromatography by exchange ions on anionic resin (53, 6, 9, 40, 49, 41), partition chromatography on Celite (42), and partition chromatography on silica introduced in 1946 by F.A. Isherwood (26).

It is undoubtedly this last procedure which has shown itself to be the most productive up to the present, but the numerous restatements which have appeared

since Isherwood's article plainly show that the method is not completely satisfactory. It seemed interesting to us to make a critical study of the chromatography methods on silica for carboxylic acids, in order to derive a method which as much as possible, would put together all the improvements brought about by the numerous restatements. The method which we will describe in the second part of this study will show to what extent we have succeeded in this attempt.

In the partition chromatography of carboxylic acids, the stationary phase is constituted by a silica-aqueous phase mixture, loaded into a column at the top of which is placed a sample of carboxylic acid. The moving phase is generally composed of a mixture of organic solvents of differing polarities. The effluent is collected in fractions of volume or of identical weight, in ml or grams, in which the eluted acids are determined.

I.--The Stationary Phase: silica and the aqueous phase. Preparation of silica

Silica can give rise to two phenomena of chromatography: a phenomenon of partition and it then acts only as a simple support of the aqueous phase, and a phenomenon of adsorption: here it intervenes actively itself. The separation of the carboxylic acids results essentially from the phenomenon of partition, and the silica doesn't have to bring about a phenomenon of adsorption. Silica is likely to absorb a large quantity of the aqueous phase, and the phenomenon of partition becomes much more preponderant as the quantity of aqueous phase absorbed increases (29), but this is not sufficient for the adsorbant properties to be eliminated (cf. page 372). It is the method of preparation of the silica which is of prime importance in the necessary elimination of the adsorbant properties.

F.A. Isherwood (26) showed that silica in contact with 10 N hydrochloric acid for 24 hours at room temperature, lost its ability to adsorb acids. Therefore, silica is prepared in the laboratory from sodium silicate (26, 35, 37, 21, 23, 31)

by precipitation to 10 N HCl; after aging for two to three weeks, it is re-treated with 10 N HCl, and it is finished by washing in water, alcohol and ether. Therefore, it is usually Mallinckrodt's silica used for chromatography, prepared according to the methods of L.L. Ramsey and W.L. Patterson (43) which is used as it is (36, 22, 19, 47, 50), or after the elimination of the finest particles (8, 55, 30) by repeated suspensions in water and drying at 100° for 24 hours. The elimination of the fine particles has the sole effect of increasing the speed of the flow, and the heating to 100° doesn't change the properties of the silica, at least not those affecting the chromatography of carboxylic acids. The various lots of Mallinckrodt silica for chromatography are far from always having identical properties (cf. page 371). Recently, silica prepared by Davison Chemical Corporation has been used, but it must be re-treated with concentrated hydrochloric acid in order to eliminate the impurities and the adsorbant properties.

Aqueous Phase

The aqueous phase is constituted by a diluted mineral acid whose role is to decrease the ionization of the acids on the column (26). 0.5 N Sulphuric acid is most often used (26, 35, 8, 23, 55, 50); also concentrations of 0.05 N (30), 0.1 N (46), 1 N (45) or 4.7 N are used. 0.01 N Hydrochloric acid was recommended in place of 0.5 N sulfuric acid in order to avoid the decomposition of the formic acid (37). Finally, there are still methods where the aqueous phase is water (36, 47). However, it is certainly true, that the use of diluted mineral acids gives much better results: the "peaks" of acids are more symmetrical, sharper and don't end by "dragging."

Some mixtures of the solvent used as the moving phase must be in equilibrium with the aqueous phase of the silica column; to do this, they are shaken in a decantation phial with a known volume of the aqueous phase. They thus absorb a

certain quantity of the mineral acid, varying with the nature of the acid--the least with sulfuric acid for the normal butanol-chloroform mixture (26) and more, the more concentrated is the acid solution. The mineral acid thus present in the elution solvents is found again in the effluent and is superimposed on the organic acids. The acidity from the mineral acid must obviously be as weak as possible. This is the case with solutions of sulfuric acid of low concentrations or equal to 0.5 N. With oxalic acid in which ionization is not totally suppressed even in the presence of an acid aqueous phase, 1 N sulfuric acid just barely gives better results than 0.5 N sulfuric acid. The use of sulfuric acid has another advantage; in the course of the elution process, there comes a time when the mineral acid which is a constituent of the stationary phase is itself eluted, and eventually, at the same time as one of the last organic acids; sulfuric acid is one of the mineral acids which leaves the column last. In our work, we have used 0.5 N sulfuric acid; it leaves the column well after the tartaric acid, which is, among the carboxylic acids from C_1 to C_6, the one which is eluted last.

Quantity of Aqueous Phase Per Gram of Silica

In order to make the stationary phase, a certain volume of the aqueous phase is added to a given quantity of silica. After mixing, a powder is obtained which must not stick to the sides of the receptacle, and which must give an homogenous suspension when shaken with chloroform which is to be used to transfer the mixture into the chromatography tube. If the quantity of the aqueous phase added is too great, the silica is not capable of adsorbing it completely; a damp powder is obtained which sticks to the sides and splits apart when chloroform is added; the transfer into the column is very difficult and the silica doesn't pile up simply by the passage of the chloroform. It is recommended to add per gram of silica, the maximum quantity of the aqueous phase which allows a dry powder to be formed.

This quantity varies with the silica used. The silica prepared according to Isherwood (26) or Marshall and colleagues (35) or coming from the Davison Chemical Corporation (45) is likely to absorb its weight of the aqueous phase while remaining dry: it can hydrate itself to 100%. Mallinckrodt's silica can only hydrate itself to about 75%.

Also, silica always contains a certain quantity of initial water. The total quantity of liquid which silica can contain without becoming damp is the sum of the water initially present and of the aqueous phase which is added. This sum is nearly constant and represents, for Mallinckrodt's silica, 90% of the anhydrous silica (10). The optimum quantity of the aqueous phase which should be added is therefore that which leads to the degree of hydration of the silica of 90%, taking into account the water initially present. But this water, which may be as much as 20% of the weight, varies according to the lots; for each lot therefore, it is necessary to determine the quantity of the aqueous phase which should be added (36, 18). L.W. Ferris established a formula, which enables us, for Mallinckrodt's silica, to calculate this quantity: $V = P\ (1.9\ A-1)$.

V = volume of the aqueous phase to add per gram of silica; P = weight in g of the silica used to prepare the column; A = weight attained by 1 g of silica after warming in a red hot capsule for 10 minutes.

The use of such a formula is called for above all when there is a desire to obtain in several laboratories identical results with different lots of silica. Otherwise, it suffices to determine once for a given lot the quantity of the aqueous phase to add to 10 g of silica; for example, in order that the powder just begins to stick together and cling to the sides, then cut out a few tenths of a ml of this volume. It is essential to always use with a given lot of silica, the same volume of the aqueous phase per g of silica, in order to obtain reproducible results. Actually, the volume of the solvent necessary to elute an acid

is a function of the degree of hydration of the silica: this volume increases with the degree of hydration (8, 19).

In certain methods where carboxylic acids are introduced on the column in a small volume of the aqueous phase (55, 50), it is recommended that the columns be constituted as under-saturated in order that they will better absorb the aqueous sample of acids.

To summarize, although a variation in the degree of hydration between 30% and 70% doesn't bring about significant modifications (8), generally the maximum volume of the aqueous phase is used.

Quantity of silica used for a separation
Height of the silica column

The weight of the silica used to prepare a column varies greatly according to the methods (Table I). For a column of a given diameter and an eluent of constant composition, and given a variation of the quantity of the stationary phase, formerly described as the height of the silica column, there is a proportional variation in the volume of the eluent necessary to elute an acid: when the height of the column is increased, it is necessary to increase in the same proportions the volume of the eluents, and the different acids are eluted in a greater volume of effluent; the peaks are flattened and their base is enlarged. This is why increasing the height of the column brings about only a small improvement in the quality of the separation (8).

In the case of an eluent of varying composition, but in which the polarity increases continually (15), it can be shown that with a column of given diameter: 1) the peaks are as much high and sharper as the column of silica is shorter and as the volume of the necessary eluent is smaller; 2) by contrast, the separation between acids is more efficient as the column is longer and the volume of the eluent is greater. Therefore, one cannot have simultaneously a very efficient

separation and very sharp peaks. The best height of the column is therefore the one which assures an efficient separation and gives peaks sharp enough for elution to take place in a small enough volume of effluent.

However, in the case of a mixture in which the polarity progressively increases (15), a relationship can be established between the speed of the increase of the polarity, the ability to separate and the mass of silica gel (34).

Diameter of the silica column

Table I indicates the diameters most frequently used. For a mixture of a given solvent and a given height of the silica column, the volume of the mixture necessary to elute an acid is directly proportional to the surface of the section of the column. Therefore, when one wants to separate large quantities of acid, a column of large diameter must be used (8). For a given quantity of silica, and a given mixture of the solvent, the volume of the eluent increases when the diameter of the column is smaller; for example, it takes 120 ml of a mixture of tertiary butanol (8%) and chloroform (92%) with a column 12 mm in diameter; it takes for columns of 10 mm or 7 mm diameter, 130 ml or 145 ml respectively. Just as there exists an optimum height for the silica column, conceivably, there exists an optimum diameter: with columns having diameters which are too big, the ability to separate is poor, with small diameters, the volume necessary for elution is too great. At the optimum diameter and height, the optimum amount of the stationary phase corresponds to a given quantity of silica.

Table I

Références (1)	Diamètre de la colonne (mm) (2)	Origine de la silice (3)	Poids P de silice (g) (4)	Volume V de la phase aqueuse (ml) par P de silice (5)	Degré d'hydratation $\frac{V \times 100}{P}$ (6)
F. A. Isherwood (26)	3	Isherwood	3	3 (SO_4H_2 0,5 N)	100
L. M. Marshall (35)	8	Marshall	3	3 (SO_4H_2 0,5 N)	100
A. C. Neish (37)	15	Neish	3	5,4 (ClH 0,01 N)	150
Marvel et Rand (36)	18	Mallinckrodt	20	12 (eau)	60
E. J. Robert et L. E. Martin (47) C. E. Frohman et coll. (21)	8	Marshall	3	1,15 (SO_4H_2 4,7 N)	38
W. A. Bulen et coll. (8)	12	Mallinckrodt	8	5,5 (SO_4H_2 0,5 N)	69
	7		12	8,5 (SO_4H_2 0,5 N)	71
F. G. Houston et J. L. Hamilton (23)	13	Isherwood	3	3 (SO_4H_2 0,5 N)	100
Rice et Pederson (46)	17	Mallinckrodt (?)	20	11,5 (SO_4H_2 0,1 N)	57
V. Zbinovsky et R. H. Burris (55)	10	Mallinckrodt	4	2,4 (SO_4H_2 0,5 N)	60
R. W. Scott (50)	8	Mallinckrodt	1	0,5 (SO_4H_2 0,5 N)	50
K. O. Donaldson et coll. (15)	10	Isherwood	3	2 (SO_4H_2 0,05 N)	67
Kinnory et coll. (30)	11	Mallinckrodt	8,8	5,4	61
Resnik F. E. et coll. (45)	10 (?)	Davison Chem. corp.	4	4 (SO_4H_2 N)	100
Ladd et Nossal (31)	6	Isherwood	1,1	0,8 (SO_4H_2 0,5 N)	73

1-References; 2-Diameter of the column (mm); 3-Origin of the silica; 4-Weight P of silica (g); 5-Volume V of the aqueous phase (ml) per P of silica; 6-Degree of hydration

Experimental Section
II. Study of the quality of silica

In the course of our work, we have verified that there are great differences in quality between various lots of Mallinckrodt silica. We used three different lots of silica: lot YDR (control number), lot XNB-1, and lot ACM. Silica of lots YDR and XNB-1 gave us very good chromatographies, as good using the method of W.A. Bulen and colleagues (8) (fig. 1), as with that proposed in the second paragraph of this article: acids well separated, eluted in a small volume of the moving phase; symmetrical and sharp peaks; base of the peaks, narrow. On the other hand, silica lot ACM didn't enable us to perform correct separations: acids left the column "dragging," eluted in a volume of solvent three to four times greater; unsymmetrical and flattened peaks; bases of the peaks are very wide (figure 2).

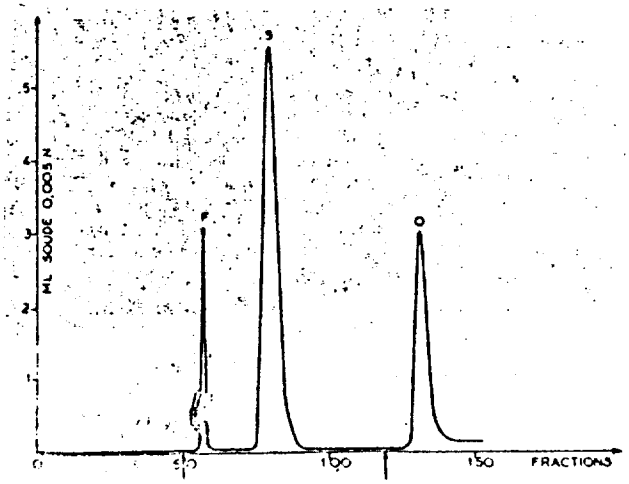

Figure 1 - Chromatogram of fumaric acids (F), succinic acids (S) and oxalic acids (O) obtained using the method of Bulen and colleagues (8), with Mallinckrodt silica, lot YDR.

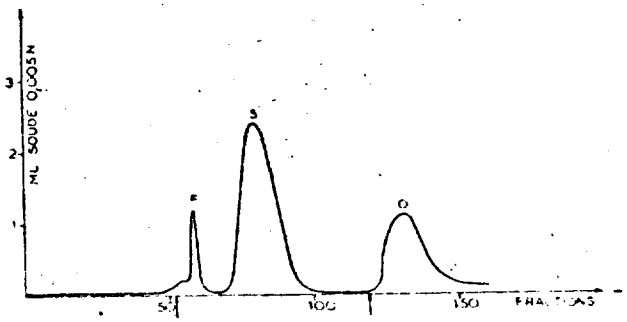

Figure 2 - Chromatogram of fumaric acids (F), succinic acids (S) and oxalic acids (O) obtained using the method of Bulen and colleagues (8), with Mallinckrodt silica lot ACM.

We therefore tried to find under what conditions we could make this silica (ACM) usable. To do this, we used the method of W.A. Bulen (8), limiting ourselves to fumaric, succinic and oxalic acids; in all our attempts, we always introduced at the top of the column the same quantity of each acid.

E.W. Malmberg (33) has recently studied the influence of the size of the silica particles on the chromatographic properties. He pointed out that certain silica, not necessarily Mallinckrodt, recently put on the market, is very different from previous silica, as much in relation to the size of the particles as to the chromatographic properties in general; the old silica gave better results. Silica lot ACM is more recent than silica lots XNB-1 and YDR. We therefore made two attempts, one using silica lot ACM as it is found commercially, another after the elimination of the finest particles by repeated suspensions in water, then drying at 100° for 24 hours (8). The separations obtained were as poor in one case as in the other; therefore, the size of the particles, at least between certain limits, doesn't interfere in the partition chromatography of carboxylic acids.

Subsequently, we tried to improve the separations by modifying the volume of the aqueous phase (5 N H_2SO_4). Instead of adding 5.5 ml of 0.5 N sulfuric acid to 8 g of lot ACM silica, we took respectively, at the time of two separate attempts, 5.00 ml (fig. 3) and 5.80 ml (fig. 4); with 5.9 ml of cracked silica. Looking at figures 3 and 4, it can be seen that with silica lot ACM, a slight variation of the aqueous phase has a great influence on the speed of the separation, in contrast to what took place with the better silica. The greater the aqueous volume, the more slowly the acids are eluted, and in equal proportions. The peaks of figures 3 and 4 are far from being symmetrical; the peaks corresponding to succinic and oxalic acid both have the appearance of reflecting the presence of two acids incompletely separated. Therefore the separations cannot be improved by modifying the volume of the aqueous phase.

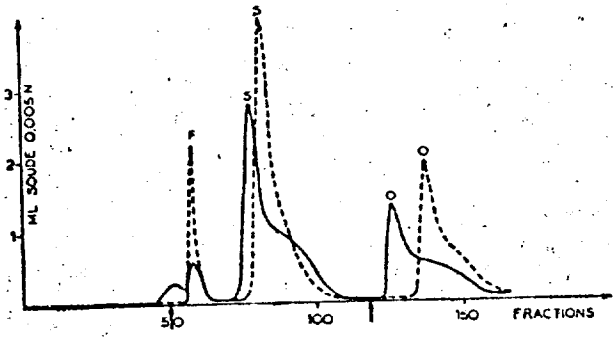

Figure 3 and 4 - Chromatogram for fumaric acid (F), succinic acid (S) and oxalic acid (O) obtained with Mallinckrodt silica lot ACM, using Bulen and colleagues' method modified: 5 ml 5N H_2SO_4 (solid line), and 5.8 ml 5 N H_2SO_4 (dotted line) for 8 g silica.

G.B. Corcoran (12) has indicated very recently that Mallinckrodt's silica is not good as the support of the aqueous phase because of its adsorbant properties, and he also treats the silica with 10 N hydrochloric acid to eliminate them. We have subjected silica lot ACM to this treatment and chromatographed the acids on the silica thus treated: the results were not good.

Also, F.E. Resnik and colleagues (45) who used silica from the Davison Chemical Corporation, recommended a treatment with concentrated hydrochloric acid for one reason in order to eliminate the cations which the soluble salts may form with carboxylic acids, and for another reason to suppress the adsorbant properties. We have thus treated silica lot ACM according to the technique of Resnik and colleagues and we used in two attempts differing from each other in the volume of the aqueous phase which is respectively 6.2 ml (fig. 5) and 6.6 ml (fig. 6) for 8 g of silica. With 6.7 ml, the silica sticks to the sides and cracks (silica not treated with HCl cracks with 5.9 ml; it contains initially more water). Looking at figures 5 and 6, it can be seen that the peaks of succinic and oxalic acid are

composed of two parts separated by an angular point. This clearly translates the manifestation of two distinct phenomena: a phenomenon of partition and a phenomenon of adsorption; the part of the peak situated to the left of the angular peak is interpreted mainly as a phenomenon of partition, that part at the right principally reflects adsorption.

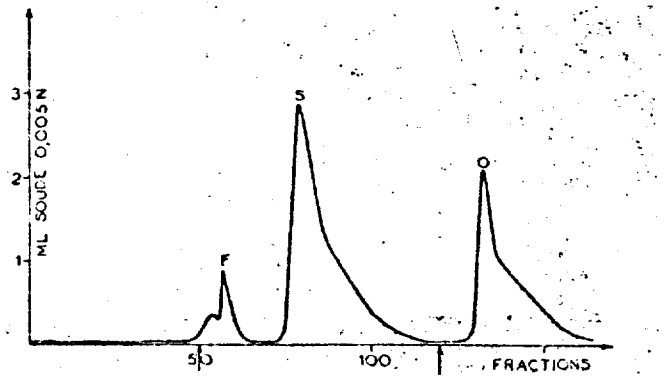

Figure 5 - Chromatogram for fumaric acid (F), succinic acid (S) and oxalic acid (O) obtained using Bulen and colleagues' method with Mallinckrodt silica lot ACM, treated according to Resnik (45); 6.2 ml 0.5 N H_2SO_4 for 8 g silica.

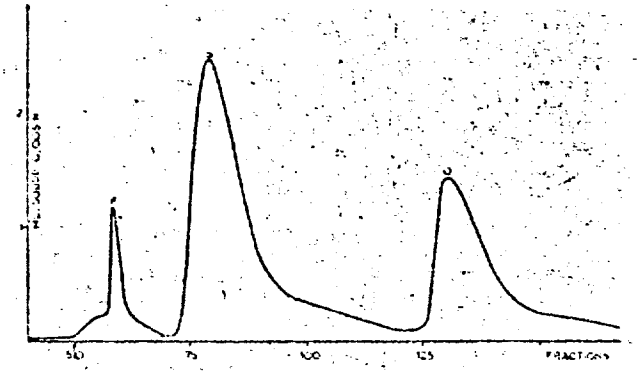

Figure 6 - Chromatogram for fumaric acid (F), succinic acid (S) and oxalic acid (O) obtained using Bulen and colleagues' method with Mallinckrodt silica lot ACM treated according to Resnik (45); 6.6 ml 0.5 N H_2SO_4 for 8 g silica.

The same remarks can be made for the corresponding peaks in figures 3 and 4. For each of these peaks, the percentage of the acid eluted before and after a particular point can be calculated. The results are shown in Table II.

Table II

	(1) Quantité de SOH 0,5 N pour 8 g de silice	(2) Acide succinique (3) % d'acidité éluée du début au point particulier	(2) Acide succinique (4) % d'acidité éluée du point particulier à la fin	(5) Acide oxalique (3) % d'acidité éluée du début au point particulier	(5) Acide oxalique (4) % d'acidité éluée du point particulier à la fin
(6) silice traitée 24 heures à 100° C	5,0 ml (fig. 3)	64	36	52	48
	5,8 ml (fig. 4)	77	23	75	25
(7) silice traitée d'après Resnik (37)	6,2 ml (fig. 5)	68	32	52	48
	6,6 ml (fig. 6)	80	20	78	22

1-Quantity of 0.5 N HOS for 8 g silica; 2-Succinic Acid; 3-% of acidity, eluted at the beginning at a particular point; 4-% of acidity, eluted at the end at a particular point; 5-Oxalic Acid; 6-Silica treated for 24 hours at 100°C; 7-Silica treated according to Resnik (37).

It can be seen from this table that the percentage of the acid eluted before a particular point is higher for the trials where the aqueous phase is greater. In other words, here is evidence that the greater the volume of the aqueous phase for a given weight of silica, the more important is the phenomenon of partition, and the more it detracts from the phenomenon of adsorption, but still without it being suppressed.

Finally, with the silicas which we treated again, we have also done chromatographies by taking for the aqueous phase, in place of 0.5 N sulfuric acid, this same acid in a stronger concentration (1 N) or weaker (0.1 N); we were never able to get improvements.

To summarize, among the various lots of Mallinckrodt silica, certain ones (YDR, XNB-1) constitute good supports for the aqueous phase, others (ACM) are

unusable even after various treatments with concentrated hydrochloric acid. Perhaps, two or three successive treatments of concentrated hydrochloric acid are necessary. It is simple to see which of the categories of silica have been used when doing a control separation of two or three acids; if the silica is right, peaks similar to the ones in figure 1 will be formed.

Figure 7 shows the result of a separation done with silica prepared from sodium silicate following the method of L.M. Marshall and colleagues (35) and using 8 ml of 0.5 N sulfuric acid for 8 g silica, the other conditions being those of the method of W.A. Bulen (8). In comparing the peaks of figures 1 and 7 corresponding to the same acids, it can be seen that the silica prepared according to Marshall lends itself well to the chromatography of carboxylic acids, but it gives less sharp peaks than certain lots of Mallinckrodt's silica.

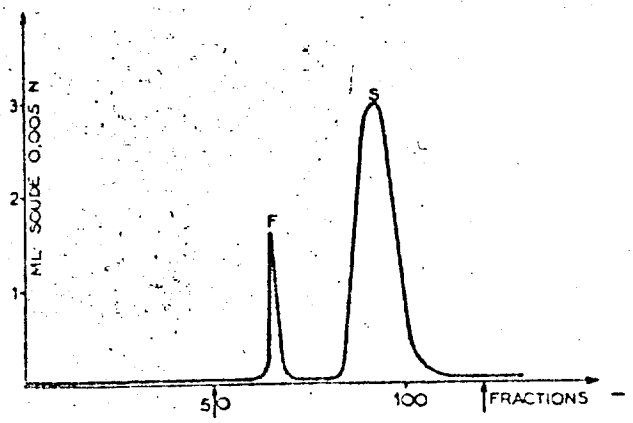

Figure 7 - Chromatogram for fumaric acid (F) and succinic acid (S) obtained using the method of Bulen and colleagues with silica prepared according to Marshall (34).

III. Introduction of Carboxylic Acids on the Silica Column

The second part of this work describes a method for the preparation of the silica column.

There exist several procedures for the introduction of carboxylic acid on to the top of the column:

1. The acid sample is dissolved in 1 or 2 ml of a mixture of tertiary amyl alcohol-chloroform (26, 35), tertiary ether amyl alcohol (37) or normal butanol-choroform (36) which is put at the top of the column.

2. The sample is dissolved in 0.5 ml (for example) of the aqueous phase and 1 g of silica is added; chloroform is added to the mixture, and it is poured onto the silica already in the chromatography tube.

3. The sample, dissolved in a very small volume of the aqueous phase (0.15 ml or less), is introduced directly with a micropipette at the top of the silica column purposely undersaturated with the aqueous phase (55).

4. The sample of acid may be absorbed by using a very porous round paper filter whose diameter is equal to the interior diameter of the tube; the dried paper is pushed down onto the top of the silica column; the acids are put into the column with a few tenths of a ml of organic solvent (55, 30) or with an even smaller (0.05 ml) volume of the aqueous phase (50).

It seems that the method of introducing the acids onto the column with organic solvents has been abandoned because of the great differences in the solubilities of the carboxylic acids in the solvents. In order to transfer a large quantity of acids onto the column, the use of silica is very common and good quantitative results are obtained. The procedure using the paper circle works well for small quantities of acid; it may be advantageous to use when trying to chromatograph on the silica two unseparated acids without a preliminary chromatography. The introduction of the acids onto the column in the aqueous phase has certain advantages:

the salts of the volatile acids introduced in this way may be hydrolyzed by the addition of sulfuric acid onto the same column, and the losses will be very limited (55).

IV. The Moving Phase and Elution

The mixture of solvents

Mixtures of normal butanol (26, 35, 36, 8, 50 46, 28), of tertiary butanol (45), of normal amyl alcohol (15), tertiary amyl alcohol (35, 21), or of normal amyl normal tertiary alcohols (34, 31) for one part and chloroform for the other part are principally used. Isobutyl methyl ketone (24) and ether have been used unmixed as other solvents.

Succinic and lactic acids are not separated when eluted with the normal butanol-chloroform mixture; however, the normal butanol-aromatic carbide mixture (37) and particularly, the normal butanol-benzene mixture (37, 8, 52) bring about the separation of succinic, lactic and α-ketoglutaric acids (30); it works less well for citric, isocitric, tartaric and even malic acids.

The use of normal butyl alcohol may be criticized because in view of the large reacting surface and the acidity of the column, it may produce between the alcohol and certain carboxylic acids, especially oxalic acid, an esterification (8, 45) which is more significant, the slower the flow of the solvent. Tertiary butyl alcohol and tertiary amyl alcohol are difficult to esterify under the conditions of the column and don't present this problem. Tertiary butyl alcohol has the additional advantage of enabling titration to take place in the presence of only one phase, and the process is therefore easier (45).

In order to avoid losses due to esterification, mixtures where ketones replace the alcohols have been proposed--in particular, methyl isobutyl ketone-methylene chloride mixture, the methylene chloride having the advantage over chloroform of

leading to more acute peaks. But up to now, separations done using ketones haven't seemed to be as efficient as those obtained using alcohols: succinic and lactic acids were not separated, nor were citric and isocitric acids.

Some authors (47) haven't found the risk of esterification to be a source of difficulty. However, to be able to separate equally, it is preferable to use a system of elution which avoids esterification. Following Resnik and colleagues, we chose a tertiary butanol-choloroform mixture which didn't lead to losses from esterification, and contrary to what has been thought (47), gives separations at least as efficient as normal butanol (cf. second part).

Elution

To accomplish the elution, a certain number of solvents may be used, introduced onto the column one after the other, each mixture being distinguished from the preceding one by a higher concentration, constituting a higher polarity (alcohol, ketone, ether, ...); thus there is a progressive increase, but discontinuous polarity, since only one mixture whose concentration is the most polar increases progressively, but not in a continuous manner.

a. Progressive but discontinuous increase of polarity.

This is the procedure most often used (36, 21, 8, 23, 46, 47, 50, 45, 28). Its application is very flexible--the volume and composition of the mixtures may be varied as desired, and the method adapted to the substance to be analyzed or to the mixture of acids. But the method may be criticized because of the necessity of close observation at the time when the solvents are changed when this is done manually, in order that at the instant when the mixtures are used up, no air enters into the column.

Recently, some apparatus has been described which enables the changing of the solvents to be done automatically (1, 48) and the method may be adapted to the work routine.

b. Progressive and continuous increase of polarity.

The mixtures for continuous increase of polarity are obtained from "mixing chambers". In the apparatus of K.O. Donaldson and colleagues (15), the polar compound (normal butanol or normal amyl alcohol) is introduced into the bottom of a mixing chamber filled with chloroform, where the two liquids mix progressively because of their differences in density. R.M. Bock and colleagues (2) described and studied various mixing chambers. When such a device is used, certain solvents cannot be put into equilibrium with the aqueous phase (48). Although, the speed of increase of polarity may be varied, the law of the variation of polarity is fixed once and for all, and from this comes a certain rigidity analogous to that which results from the use of automatic devices for changing the solvent. K.O. Donaldson and colleagues' device (15) used with the normal amyl alcohol mixture plus tertiary amyl alcohol-chloroform (34) or with the ether-benzene (30) mixture brought about the separation of succinic, lactic and α-ketoglutaric acids.

Flow Speed of the Effluent

The flow speed must not be too slow with esterifiable solvents; it should generally be 2 to 3 ml/mn.

It is dependent on:

a) the nature of the silica; b) the size of the particles; c) the height of the silica column; d) the degree of compression of the silica; e) the presence of interfacial air-liquid tension, the presence of air in the column slowing down the flow (44); f) the degree of hydration of the silica, the flow speed increasing with the quantity of aqueous phase per gram of silica (19); g) the weight and then the height of the column of liquid which rises above the silica; h) the nature of the solvents used; the speed decreases gradually as the concentration of alcohol in alcohol-chloroform mixtures increases. With mixtures having a ketone base, it

is less, the greater the molecular weight of the ketone (50).

The flow speed may be increased, most often by exerting pressure of nitrogen or compressed air on the column, or by increasing the height of the liquid column which rises above the silica column. When pressure is created by means of compressed gas, it is advisable to have a manostat, a manostat for the escape of gas for example.

Volume of the Fractions Collected

In the great majority of cases, factions of from 2 to 5 ml are collected.

V. Determination and Characterization of the Acids after Separation

In general, the carboxylic acids are determined by titration, but the polycarboxylic acids may also be determined fluorimetrically.

Titrimetric Method

The acids are titrated in their respective parts to which are added a few milliliters of water, generally by diluted soda (0.01 N or 0.005 N), rather than by baryta (23), in the presence of red phenol which gives clearer toning than thymol blue (26, 35).

Towards the end of the titration, it is necessary to vigorously shake the immiscible mixture of the organic solvent and water in order to make the untitrated acids of the organic phase go into the aqueous phase. The shaking can be done by means of a stream of air of free CO_2, but when the titration is being done in tubes, it is simpler and also better to do the shaking by hand, stopping the tube with a rubber stopper.

When the effluent is a tertiary butanol-chloroform mixture, after the addition of a few milliliters of water, the tubes may be placed in a vapor bath in order

to get rid of the chloroform whose departure leaves a liquid of only one phase (45), weaker to titrate. We have found that titration in the presence of two phases presents no difficulty, and we have never eliminated the chloroform.

G. Jolchine (28) titrated in the presence of lauryl sulfonate and sodium which assures, upon shaking, a close contact between the two phases. However, when the two phases form a stable enough emulsion, the colorations coming from the color indicator are paler and sometimes the toning is less perceivable. This is why, in the presence of malic and especially citric acid whose coefficients of separation are clearly in favor of the aqueous phase, we avoid the formation of an emulsion by only shaking lightly (18) as long as the aqueous phase remains yellow (phenol red indicator); when the color changes to violet, we shake it vigorously; one or two drops of 0.005 N soda suffice to finish the titration.

Fluorimetric Method

It is rare that the sample introduced into the column doesn't contain mineral acids as well as organic acids. Mineral acids are also eluted, although slowly, and they may be superimposed onto certain polycarboxylic acids. Thus, nitric acid may be eluted at the same time as isocitric acid. This is in part why C.F. Frohman and J.M. Orten (20) used a fluorimetric method for the determination of polycarboxylic acids after chromatographic separation. It is based on the formation of fluorescent derivatives in the presence of resorcinol. The intensity of the fluorescence is measured in a fluroimeter; the color of the fluorescence gives information on the nature of the acids. Further, in cases where the acids contain marked atoms, their fluorescent derivatives may be extracted by ether in which they are soluble, in view of their radioactive properties.

Quantitative Considerations--Sensitivity

Recovery is good when the silica is pure and not adsorbent. It attains at least 90 to 95% and often the carboxylic acids are determined to within 5%. For oxalic acid, eluted by an esterifiable solvent, the recovery is only 85 to 90%. When the blank runs are low, one can reveal and estimate up to 1 to 2 μeq. of acid.

Characterization of the Separated Acids

When, from one separation to another, one always operates under the same conditions, the results are very reproducible, and the individual acids of certain groups of acids are always eluted in the same amounts of effluent, within a few milliliters. The location of a peak on a chromatogram therefore allows certain presumptions to be made about the nature of the corresponding acid. But several acids may be eluted in the same amounts and give only one peak and an undetermined acid may be eluted in the same amounts as an acid generally considered to be at this place. From this comes the necessity of characterizing the place where the acids correspond to each peak by paper chromatography, by specific reactions, by formation of characteristic derivatives, by the equivalent of neutralizing, ...

a. Paper chromatography.

Chromatography may be used in one (28, 45) to two dimensions (45, 14) or circular chromatography (5) which is faster than either two or one dimensional. It is true that by the addition to the solvents of 8 quinoline, for example (45), the stains on the paper can be made fluorescent and can be observed under ultraviolet light even before drying, and wouldn't prevent them from then showing up normally (17, 39).

b. Specific reactions.

Formation of fluorescent compounds by the reaction between the polycarboxylic

acids and, the resorcinol (20), between malic acid and β-naphtyyl (28), citric acid and thiomyl chloride (28). Transformation of isocitric acid into lactone, under a vacuum in a double-boiler at 100° for two hours, then paper chromatography (11); we brought about more simply the transformation of isocitric acid into lactone directly on paper (cf. second part). Specific enzymatic degradation and characterization of the products of degradation, etc.

VI. Remarks Concerning Certain Groups of Acids. Limits of the Method. Comparison of Partition Chromatography and Ion-Exchange Chromatography

Volatile Acids

It is certain that the volatile acids are separated and determined much better by gas-liquid partition chromatography according to the method A.T. James and A.J.P. Martin (27). It is advantageous to determine them using this method, which is slightly more sensitive. Therefore, they can be eliminated from the extract of acids; under these conditions, they don't interfere in chromatography on silica of non-volatile acids.

Ketonic Acids

Pyruvic and α-ketoglutaric acids, more stable than other ketonic acids, may be determined easily enough by chromatography on silica.

Oxalic and Glycolic Acids

These acids are not separated by the normal butanol-chloroform mixture nor by the methyl isobutyl ketone-methylene chloride mixture (50). The partially ionized oxalic acid often leaves the column more or less "dragging."

Glycolic and Tricarballylic Acids

They are not separated by the tertiary butanol-chloroform mixture; they are separated with normal amyl alcohol- or tertiary amyl alcohol-chloroform mixture (31).

Citric and Isocitric Acids

They are not separated by the benzene-ether mixture (30) or the methyl isobutyl ketone-methylene chloride mixture. They are separated by normal butanol-chloroform and tertiary butanol-chloroform mixtures.

Quinic Acid

A.C. Hulme (24) didn't succeed in separating this acid in a satisfactory manner with either methyl isobutyl ketone- or normal butanol-chloroform mixtures.

Mineral Acids

Although it takes place slowly, the mineral acids end up being eluted themselves. Nitric acid is often eluted with isocitric acid. Phosphoric and hydrochloric acids may obstruct the determination of tartaric acid. The mineral acids leave the column in the form of flattened, irregular peaks. Sulfuric acid is eluted well after tartaric acid when its concentration in the aqueous phase of silica containing the sample is not too great (H_2NSO_4 and free acids). Generally, it begins to be eluted when the level of hydration reaches mid-column.

Comparison of the Chromatography of Carboxylic Acids on a Silica Column (partition chromatography) and on a Anionic Resin Column (ion-exchange chromatography)

Resin columns have the advantage over silica of having a greater capacity for absorption of acids; with resin columns, the acids may be fixed on a great height of the column, while with silica columns, the acid sample must occupy a narrow band at the top of the column. In order to make use of this advantage, J.K. Palmer (4) recently described a method of separating carboxylic acids on a strongly basic anionic resin (Dowex 1, X-10), which can be considered an improvement in comparison to the previous methods of chromatography on a resin. However, in this method, the quantity of acids actually introduced onto the resin columns (0.2 m.eq.) seems to be of the same magnitude as that used with silica columns. It can be seen in the second part of this work that 1.0 m.eq. and more of acids may be easily introduced on a silica column of little height. When a resin column is used, it isn't necessary that the volume of the sample introduced on the column be very small (2 to 4 ml). On the other hand, with a silica column, this volume must be small, and as a result, when it is desired to chromatograph relatively large quantities of acids, the solution of the sample must be fairly concentrated. Here is a difficulty which, however, may be easily surmounted by using a small amount of silica (8) to transfer the aqueous sample (0.5 to 1.0 ml) onto the column (cf. higher).

In Palmer's method, malic, citric and tartaric acids leave the resin column fairly early, and their peaks are sharp; by contrast, these acids are eluted last on silica columns and the peaks are generally flattened. But it can be seen from part two that with the method which we used, very good peaks were obtained for these acids. The determination of these acids after separation is easier

after chromatography on silica, since, very generally, it suffices to titrate the acids in various amounts (the presence of two phases is not a significant obstacle for the accuracy of the titration), while for certain acids after separation on resin, it is necessary to use procedures other than simple titration (41).

It has been shown that there are variations among different lots of resin (41), just as there are variations among different lots of silica. The separation of acids is more rapid on a resin column than on a silica column, and when one is particularly interested in malic and citric acid out of the total acid sample, it may be interesting to use Palmer's method (41). The resin columns may be regenerated while the silica columns must be reconstituted before each separation.

In summary, it seems that in the actual state of the art, certain methods of partition chromatography on silica allow, in spite of everything, a more final work than the techniques of chromatography on resin: a greater number of acids separated from a single operation, more efficient separation of the acids, easier and faster determination of the acids after separation. The two methods may sometimes complement each other; for example, in a biological substance, separation of the "acid" part from the "neutral" part by fixation of the acids on a strongly basic anionic resin in the form of formate, after having fixed the "basic" part on a cation resin; total elution of the acids in light of the total acidity, or partial elution in light of the separation of certain acids or groups of acids, by a small volume (100 ml) of 6 N formic acid (38, 41); then further separation of the acids on the silica column and subsequent determination.

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N° 48. — La séparation et la détermination des acides carboxyliques de C_1 à C_6 par chromatographie de partage sur colonne de silice, I. — Étude générale,

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Les divers facteurs qui interviennent dans la chromatographie sur colonne de silice des acides carboxyliques de C_1 à C_6 ont été étudiés.

La qualité de la silice est le point le plus important à considérer. Des lots de silice de même provenance et de même dénomination commerciale peuvent présenter entre eux, des différences parfois considérables en ce qui concerne les propriétés adsorbantes. Les silices qui manifestent des propriétés adsorbantes sont inutilisables et il est souvent difficile, sinon impossible d'éliminer ces propriétés néfastes. Quand on dispose de plusieurs lots de silice commerciale, différant par le numéro de fabrication il y a intérêt à effectuer avec chacun d'eux une séparation témoin afin de reconnaître les silices adsorbantes et de les rejeter. Par exemple la silice Mallinckrodt XNB-1 et YDR est excellente, celle numérotée ACM est mauvaise.

La chromatographie sur colonne présente, par rapport à la chromatographie sur papier l'avantage de permettre la séparation et la détermination de grandes quantités d'un corps à côté de très petites quantités d'un autre composé. Dans la séparation et la détermination des acides organiques par chromatographie sur colonne, on peut discerner trois grands procédés : la chromatographie par échange d'ions sur résine anionique (53, 6, 9, 40, 49, 41), la chromatographie de partage sur Célite (42), et la chromatographie de partage sur silice introduite en 1946 par F. A. ISHERWOOD (26).

C'est indubitablement ce dernier procédé qui s'est montré le plus fécond jusqu'à présent, mais les nombreuses mises au point qui ont paru depuis le mémoire d'ISHERWOOD montrent bien que la méthode ne donnait pas entière satisfaction. Il nous a semblé intéressant de faire une étude critique des méthodes de chromatographie des acides carboxyliques sur silice, afin d'en déduire une méthode qui, dans la mesure du possible, réunisse les améliorations apportées par les mises au point successives. La méthode que nous exposerons dans une deuxième partie de cette étude montrera dans quelle mesure nous avons réussi dans cette tentative.

Dans la chromatographie de partage des acides carboxyliques, la phase immobile est constituée par un mélange silice — phase aqueuse, entassé en une colonne au sommet de laquelle on place l'échantillon des acides carboxyliques. La phase mobile se compose en général d'un mélange de solvants organiques de polarité différente. Les acides se partagent entre les deux phases et traversent la colonne à des vitesses différentes. L'effluent est recueilli en fractions de volume ou de poids identiques, de quelques ml ou de quelques grammes, dans lesquelles on détermine les acides élués.

I. — La phase immobile : silice et phase aqueuse.

Préparation de la silice.

La silice peut donner lieu à deux phénomènes de chromatographie : un phénomène de partage, et elle n'est alors qu'un simple support de la phase aqueuse, et un phénomène d'adsorption : elle intervient alors elle-même activement. La séparation des acides carboxyliques résulte essentiellement d'un phénomène de partage, et la silice ne doit pas donner lieu à un phénomène d'adsorption. La silice est susceptible d'absorber une grande quantité de phase aqueuse, et le phénomène de partage devient d'autant plus prépondérant que la quantité de phase aqueuse absorbée augmente davantage (29), mais cela ne suffit pas pour que les propriétés adsorbantes soient éliminées (cf. page 372). C'est le mode de préparation de la silice qui est capital dans l'élimination, obligatoire, des propriétés adsorbantes.

ISHERWOOD F. A. (26) a montré que la silice, en contact avec l'acide chlorhydrique 10 N pendant 24 h à température ambiante, perdait sa faculté d'adsorber les acides. On préparait donc la silice au laboratoire à partir de silicate de Na (26, 35, 37, 21, 23, 31) par précipitation à HCl 10 N; après un vieillissement de deux à trois semaines, on retraitait par HCl 10 N et on finissait en lavant à l'eau, à l'alcool et à l'éther. Depuis, c'est surtout la silice pour chromatographie de Mallinckrodt, préparée suivant RAMSEY L. L. et PATTERSON W. L. (43) qui est utilisée, ou bien telle que (36, 22, 19, 47, 50), ou bien après élimination des particules les plus fines (8, 55, 30) par suspensions répétées dans l'eau et séchage à 100° pendant 24 h. L'élimination des fines particules a pour seul effet d'augmenter la vitesse d'écoulement et le chauffage à 100° ne modifie pas les propriétés de la silice, tout au moins en ce qui concerne la chromatographie des acides carboxyliques. Les divers lots de silice Mallinckrodt pour chromatographie sont loin d'avoir toujours des propriétés identiques (cf. page 371). On a utilisé récemment la silice préparée par la *Davison, Chem. Corp.* (45) mais il faut la retraiter à l'acide chlorhydrique concentré pour éliminer les impuretés et les propriétés adsorbantes.

Phase aqueuse.

La phase aqueuse est constituée par un acide minéral dilué dont le rôle est de diminuer l'ionisation des acides sur la colonne (26). C'est l'acide sulfurique 0,5 N qui est le plus utilisé (26, 35, 8, 23, 55, 50); on l'utilise aussi à la concentration de 0,05 N (30), de 0,1 N (46), de 1 N (45) ou de 4,7 N (21). On a préconisé l'acide chlorhydrique 0,01 N à la place de l'acide sulfurique 0,5 N pour éviter la décomposition de l'acide formique (37). Enfin, il existe encore des méthodes où la phase aqueuse est l'eau (36, 47). Il est cependant bien certain que l'emploi d'un acide minéral dilué donne de bien meilleurs résultats : les « pics » des acides sont plus symétriques, plus aigus et ne se terminent pas en « traînée ».

Certains mélanges de solvant utilisés comme phase mobile doivent être en équilibre avec la phase aqueuse de

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la colonne de silice; on les agite pour cela dans une ampoule à décantation avec un volume déterminé de la phase aqueuse. Ils absorbent ainsi une certaine quantité d'acide minéral, variable avec la nature de cet acide, minimum avec l'acide sulfurique pour le mélange butanol normal-chloroforme (26), et d'autant plus grande que la solution acide est plus concentrée. L'acide minéral ainsi présent dans les solvants d'élution se retrouve dans l'effluent et se superpose aux acides organiques. L'acidité due à l'acide minéral doit évidemment être aussi faible que possible; on obtient alors des « à blancs » très bas. C'est le cas avec des solutions d'acide sulfurique de titre inférieur ou égal à 0,5 N. Avec l'acide oxalique dont l'ionisation n'est pas totalement supprimée même en présence d'une phase aqueuse acide, l'acide sulfurique 1 N ne donne guère de meilleurs résultats que l'acide sulfurique 0,5 N. L'emploi de l'acide sulfurique a un autre avantage; au cours de l'élution, il arrive un moment où l'acide minéral constitutif de la phase immobile est élué à son tour et, éventuellement, en même temps qu'un des derniers acides organiques; l'acide sulfurique est l'un des acides minéraux qui sortent de la colonne le plus tard. Dans notre travail nous avons utilisé l'acide sulfurique 0,5 N; il quitte la colonne bien après l'acide tartrique, qui, parmi les acides carboxyliques de C_1 à C_6, est celui qui est élué en dernier.

Quantité de phase aqueuse par gramme de silice.

Pour constituer la phase immobile, on ajoute un certain volume de phase aqueuse à une quantité donnée de silice. Après mélange on obtient une poudre qui ne doit pas coller aux parois du récipient, et qui doit donner une suspension homogène par agitation avec le chloroforme destiné à transférer le mélange dans le tube de chromatographie. Si la quantité de phase aqueuse ajoutée est trop grande, la silice n'est pas capable de l'absorber entièrement; on obtient une poudre humide qui colle aux parois et qui gélifie dès qu'on ajoute le chloroforme; le transfert sur la colonne est très difficile et la silice ne se laisse pas tasser par simple écoulement du chloroforme. Il est recommandé habituellement d'ajouter, par gramme de silice, la quantité maximum de phase aqueuse qui permette d'obtenir une poudre sèche. Cette quantité varie avec la silice utilisée. La silice préparée selon Isherwood F. A. (26) ou Marshall L. M. et coll. (35), ou à partir de la silice de la *Davison Chem. Corp.* (45) est susceptible d'absorber son poids de phase aqueuse tout en restant sèche : elle peut s'hydrater à 100 %. La silice de Mallinckrodt ne peut s'hydrater qu'à 75 % environ.

D'autre part, la silice contient toujours une certaine quantité d'eau initiale. La quantité totale de liquide que peut donc contenir la silice sans devenir humide, est la somme de l'eau initialement présente et de la phase aqueuse qu'on ajoute. Cette somme est à peu près constante et représente, pour la silice de Mallinckrodt 90 % de la silice anhydre (19). La quantité optimum de phase aqueuse à ajouter est donc celle qui amène le degré d'hydratation de la silice à 90 %, compte tenu de l'eau initialement présente. Or cette eau, qui peut représenter jusqu'à 20 % du poids, varie suivant les lots; pour chaque lot, il faut donc déterminer expérimentalement la quantité de phase aqueuse à ajouter (36, 18). L. W. Ferris a établi une formule qui permet, pour la silice de Mallinckrodt, de calculer cette quantité : $V = P(1{,}9\ A - 1)$.

V = volume de phase aqueuse à ajouter à Pg. de silice; P = poids, en g de la silice utilisée pour préparer la colonne; A = poids atteint par 1 g de silice après chauffage dans une capsule au rouge sombre pendant 15 mn.

L'utilisation d'une telle formule s'impose surtout quand on désire obtenir, dans plusieurs laboratoires, des résultats identiques avec des lots différents de silice. Sinon, il suffit de déterminer une fois pour toutes, pour un lot donné, la quantité de phase aqueuse à ajouter à 10 g de silice par exemple, pour que la poudre commence juste à s'agglomérer et à coller aux parois, puis de retrancher quelques dixièmes de ml au volume trouvé. Il est essentiel de toujours utiliser avec un lot donné de silice, le même volume de phase aqueuse par g de silice, afin d'obtenir des résultats reproductibles. En effet, le volume de solvant nécessaire à éluer un acide est fonction du degré d'hydratation de la silice : ce volume augmente avec le degré d'hydratation (8, 19).

Dans certaines méthodes où l'on introduit les acides carboxyliques sur la colonne dans un petit volume de *phase aqueuse* (55, 50), on recommande de constituer des colonnes sous-saturées afin qu'elles absorbent mieux l'échantillon aqueux des acides.

En résumé, bien qu'une variation du degré d'hydratation entre 30 et 70 % n'entraînerait pas des modifications très importantes (8), on utilise généralement le volume maximum de phase aqueuse.

Quantité de silice utilisée pour une séparation. Hauteur de la colonne de silice.

Le poids de silice utilisé pour préparer une colonne varie beaucoup suivant les méthodes (tableau I). Pour une colonne de diamètre donné et un éluant de composition constante et donnée, une variation de la quantité de phase immobile, autrement dit de la hauteur de la colonne de silice, entraîne une variation proportionnelle du volume d'éluant nécessaire pour éluer un acide : quand on augmente la hauteur de la colonne, il faut augmenter dans les mêmes proportions le volume des éluants, et les différents acides sont élués dans un volume plus grand d'effluent; les pics sont aplatis et leur base est élargie. C'est pourquoi augmenter la hauteur de la colonne n'entraîne qu'une assez faible amélioration de la qualité de la séparation (8).

Dans le cas d'un éluant de composition, non plus constante, mais dont la polarité croît de façon continue (15), on a pu montrer (32) que, avec un diamètre de colonne donné : 1) les pics sont d'autant plus hauts et aigus que la colonne de silice est plus courte et que le volume d'éluant nécessaire est plus petit; 2) par contre, la séparation entre acides est d'autant plus efficace que la colonne est plus longue et que le volume d'éluant est plus grand. On ne peut donc avoir simultanément, et une séparation très efficace et des pics très aigus. La meilleure hauteur de colonne est donc celle qui assure une séparation efficace et donne des pics suffisamment aigus pour être élués dans un volume assez faible d'effluent.

Toujours dans le cas d'un mélange dont la polarité croît progressivement (15), on a pu établir une relation entre la vitesse d'augmentation de la polarité, le pouvoir de séparation et la masse du gel de silice (34).

Diamètre de la colonne de silice.

Le tableau I indique les diamètres les plus fréquemment utilisés. Pour un mélange de solvant donné et une hauteur donnée de la colonne de silice, le volume de mélange nécessaire pour éluer un acide est directement proportionnel à la surface de la section de la colonne. Donc, quand on désire séparer de grandes quantités d'acides, il faut prendre une colonne de grand diamètre (8). Pour une quantité donnée de silice, et un mélange donné de solvant, le volume d'éluant augmente quand le diamètre de la colonne diminue; quand il faut par exemple 120 ml de mélange butanol tertiaire 8 % chloroforme 92 % avec une colonne de 12 mm de diamètre, il faut, pour une colonne de 10 mm ou de 7 mm de diamètre, respectivement 130 ml ou 145 ml. De même qu'il existait une hauteur optimum de la colonne de silice, on peut concevoir l'existence d'un diamètre optimum : avec des colonnes de diamètre plus grand, le pouvoir de séparation est mauvais, avec des diamètres inférieurs, le volume nécessaire pour éluer est trop grand. Au diamètre optimum et à la hauteur optimum, correspond la masse de

de phase immobile optimum, pour une quantité donnée de silice.

de phase mobile; pics symétriques et aigus; base des pics étroite. Par contre la silice ACM ne nous a pas permis d'effectuer des

TABLEAU I

Références	Diamètre de la colonne (mm)	Origine de la silice	Poids P de silice (g)	Volume V de la phase aqueuse (ml) par P de silice	Degré d'hydratation $\frac{V \times 100}{P}$
—	—	—	—	—	—
F. A. Isherwood (26)	3	Isherwood	3	3 (SO_4H_2 0,5 N)	100
L. M. Marshall (35)	8	Marshall	3	3 (SO_4H_2 0,5 N)	100
A. C. Neish (37)	15	Neish	3	5,4 (ClH 0,01 N)	150
Marvel et Rand (36)	18	Mallinckrodt	20	12 (eau)	60
E. J. Robert et L. E. Martin (47) C. E. Frohman et coll. (21)	8	Marshall	3	1,15 (SO_4H_2 4,7 N)	38
W. A. Bulen et coll. (8)	12	Mallinckrodt	8	5,5 (SO_4H_2 0,5 N)	69
	7		12	8,5 (SO_4H_2 0,5 N)	71
F. G. Houston et J. L. Hamilton (23)	13	Isherwood	3	3 (SO_4H_2 0,5 N)	100
Rice et Pederson (46)	17	Mallinckrodt (?)	20	11,5 (SO_4H_2 0,1 N)	57
V. Zbinovsky et R. H. Burris (55)	10	Mallinckrodt	4	2,4 (SO_4H_2 0,5 N)	60
R. W. Scott (50)	8	Mallinckrodt	1	0,5 (SO_4H_2 0,5 N)	50
K. O. Donaldson et coll. (15)	10	Isherwood	3	2 (SO_4H_2 0,05 N)	67
Kinnory et coll. (30)	11	Mallinckrodt	8,8	5,4	61
Resnik F. E. et coll. (45)	10 (?)	Davison Chem, corp.	4	4 (SO_4H_2 N)	100
Ladd et Nossal (31)	6	Isherwood	1,1	0,8 (SO_4H_2 0,5 N)	73

PARTIE EXPÉRIMENTALE

II. — Étude de la qualité de la silice.

Au cours de notre travail, nous avons constaté qu'il y avait de grandes différences de qualité entre les divers lots de silice Mallinckrodt. Nous avons utilisé trois lots différents de silice: un lot YDR (numéro de contrôle) un lot XNB-1, et un lot ACM.

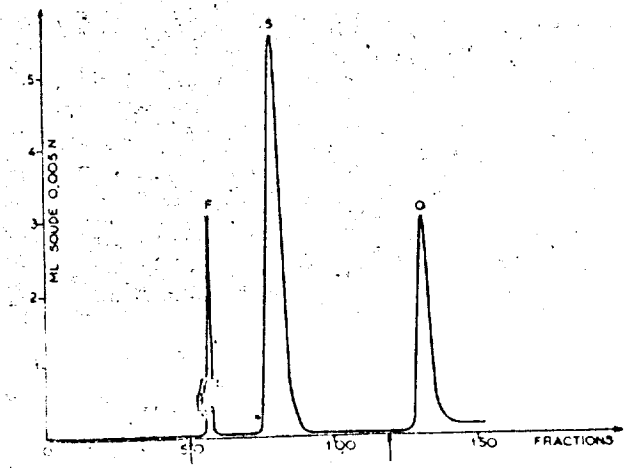

Fig. 1. — Chromatogramme des acides fumarique (F), succinique (S) et oxalique (O) obtenu d'après la méthode de Bulen et coll. (8), au moyen de la silice Mallinckrodt YDR.

Les silices YDR et XNB-1 nous ont permis de réaliser de très bonnes chromatographies, aussi bien en utilisant la méthode de Bulen W. A. et Coll. (8), (fig. 1), que celle proposée dans la 2e partie de ce mémoire : acides bien séparés, élués dans un petit volume

séparations correctes : acides quittant la colonne en « traînant », élués dans un volume de solvant trois à quatre fois plus grand; pics dissymétriques et aplatis; base des pics très large (fig. 2). Nous avons donc essayé de voir sous quelles conditions on pouvait rendre cette silice ACM utilisable. Pour effectuer ces essais, nous avons utilisé la méthode de Bulen W. A. (8), en nous limitant aux acides fumarique, succinique et oxalique; dans tous les essais, nous avons toujours introduit au sommet de la colonne, la même quantité de chaque acide.

Malmberg E. W. (33) a étudié récemment l'influence de la taille des particules de silice sur les propriétés chromatographiques. Il signale que certaines silices (pas forcément celle de Mallinckrodt) mises récemment dans le commerce sont très différentes des silices antérieures, tout aussi bien en ce qui concerne la taille des particules, que les propriétés chromatographiques en général; les silices plus anciennes donnaient de meilleurs résultats. Or,

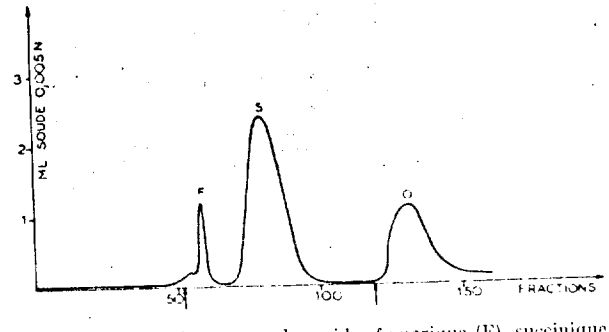

Fig. 2. — Chromatogramme des acides fumarique (F), succinique (S) et oxalique (O) obtenu d'après la méthode de Bulen et coll. (8), au moyen de la silice Mallinckrodt ACM.

la silice ACM est plus récente que les silices XNB-1 et YDR. Nous avons donc fait deux essais, l'un avec la silice ACM telle que livrée dans le commerce, l'autre après élimination des particules les plus fines par suspensions répétées dans l'eau, puis séchage

à 100° pendant 24 h (8). Les séparations obtenues ont été aussi mauvaises dans un cas comme dans l'autre; comme il fallait s'y attendre la taille des particules, du moins entre certaines limites, n'intervient donc pas, dans la chromatographie de *partage* des acides carboxyliques.

Nous avons ensuite essayé d'améliorer les séparations en modifiant le volume de la phase aqueuse (SO_4H_2 0,5 N). Au lieu d'ajouter 5,5 ml d'acide sulfurique, 0,5 N à 8 g de silice ACM, nous avons pris respectivement, lors de deux essais distincts, 5,00 ml (fig. 3) et 5,80 ml (fig. 4); avec 5,9 ml de silice gélifiait. D'après les figures 3 et 4, on voit qu'avec la silice ACM, une faible variation de la phase aqueuse a une grande influence sur l'allure de la séparation obtenue, contrairement à ce qui a lieu avec une silice correcte. Plus le volume de la phase aqueuse est grand, plus les acides sont élués tardivement, et dans des proportions assez grandes. Les pics des figures 3 et 4 sont loin d'être symétriques; les pics correspondant à l'acide succinique et à l'acide oxalique ont même l'apparence de refléter la présence de deux acides incomplètement séparés. On ne peut donc pas améliorer les séparations en modifiant le volume de la phase aqueuse.

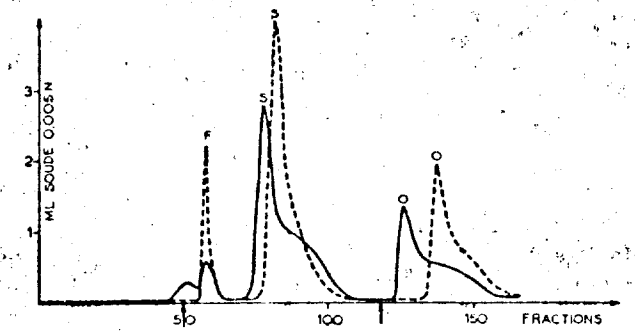

Fig. 3 et 4. — Chromatogrammes des acides fumarique (F), succinique (S) et oxalique (O) obtenus au moyen de la silice Mallinckrodt ACM, d'après la méthode de BULEN et coll. modifiée : 5 ml SO_4H_2 0,5 N (trait plein) et 5,8 ml SO_4H 0,5 N (trait pointillé) pour 8 g de silice.

G. B. CORCORAN (12) a indiqué très récemment que la silice de Mallinckrodt ne convenait pas comme support de la phase aqueuse, à cause de ses propriétés adsorbantes, aussi traite-t-il la silice à l'acide chlorhydrique 10 N pour les éliminer. Nous avons soumis la silice ACM à ce traitement et chromatographié les acides sur la silice ainsi traitée : les résultats ne furent pas meilleurs.

Par ailleurs, F. E. RESNIK et coll. (45), qui utilisent la silice de la DAVISON Chem. Corp., recommandent eux aussi un traitement à l'acide chlorhydrique concentré, d'une part pour éliminer les cations qui peuvent former des sels solubles avec les acides carboxyliques, d'autre part pour supprimer les propriétés adsorbantes. Nous avons donc traité la silice ACM suivant la technique de RESNIK et coll. et nous l'avons utilisée dans deux essais différant l'un de l'autre par le volume de la phase aqueuse qui est respectivement de 6,2 ml (fig. 5) et de 6,6 ml (fig. 6) pour 8 g de silice. Avec 6,7 ml, la silice colle aux parois et gélifie (la silice non traitée à ClH gélifie avec 5,9 ml; elle contient initialement davantage d'eau). On voit, d'après les figures 5 et 6, que les pics de l'acide succinique et de l'acide oxalique se composant de deux parties séparées par un point anguleux. Cela traduit manifestement l'existence de deux phénomènes assez nettement mis en évidence : un phénomène de partition et un phénomène d'adsorption; la partie du pic située à gauche du point anguleux traduisant surtout

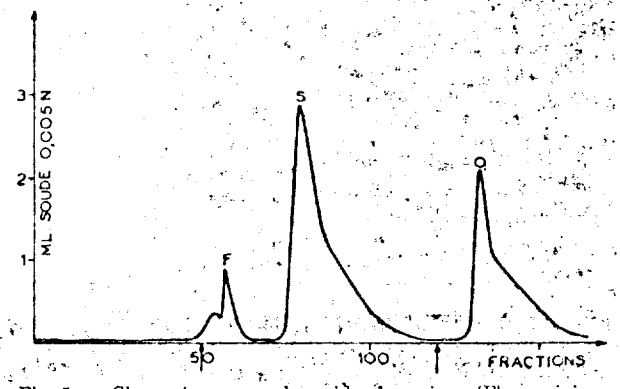

Fig. 5. — Chromatogramme des acides fumarique (F), succinique (S) et oxalique (O) obtenu d'après la méthode de BULEN et coll., au moyen de la silice Mallinckrodt ACM, traitée selon RESNIK (45); 6,2 ml SO_4H_2 0,5 pour 8 g de silice.

un phénomène de partage, celle à droite reflétant principalement une adsorption. Les mêmes remarques peuvent être faites pour

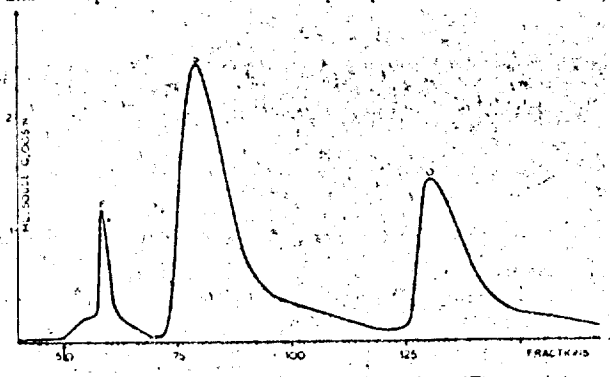

Fig. 6. — Chromatogramme des acides fumarique (F), succinique (S) et oxalique (O) obtenu d'après la méthode de BULEN et coll. au moyen de la silice Mallinckrodt ACM, traitée selon RESNIK (45); 6,6 mol SO_4H_2 0,5 N pour 8 g de silice.

les pics correspondants des figures 3 et 4. On peut calculer, pour chacun de ces pics, le pourcentage d'acide élué avant et après le point particulier. Les résultats sont consignés dans le tableau II.

TABLEAU II

	Quantité de SOH 0,5 N pour 8 g de silice	Acide succinique : % d'acidité éluée du début au point particulier	Acide succinique : % d'acidité éluée du point particulier à la fin	Acide oxalique : % d'acidité éluée du début au point particulier	Acide oxalique : % d'acidité éluée du point particulier à la fin
silice traitée 24 heures à 100° C	5,0 ml (fig. 3)	64	36	52	48
	5,8 ml (fig. 4)	77	23	75	25
silice traitée d'après RESNIK (37)	6,2 ml (fig. 5)	68	32	52	48
	6,6 ml (fig. 6)	80	20	78	22

On voit, d'après ce tableau, que le pourcentage d'acide élué avant le point particulier est plus élevé pour les essais où la phase aqueuse est la plus grande. Autrement dit, on met ainsi en évidence que plus le volume de la phase aqueuse pour un poids donné de silice est grand, plus le phénomène de partage est important, et plus il l'emporte sur le phénomène d'adsorption, sans que toutefois celui-ci soit supprimé.

Enfin, avec les silices que nous avons traitées à nouveau, nous

avons également effectué des chromatographies en prenant pour phase aqueuse, au lieu de l'acide sulfurique 0,5 N, ce même acide à concentration plus forte (1 N) ou plus faible (0,1 N); nous n'avons jamais obtenu d'améliorations.

En résumé, parmi les divers lots de silice de Mallinckrodt, il y en a certains (YDR, XNB-1) qui constituent de bons supports pour la phase aqueuse, d'autres (ACM) qui sont inutilisables, même après divers traitements à l'acide chlorhydrique concentré. Peut-être faudrait-il effectuer deux ou trois traitements successifs à l'acide chlorhydrique concentré. Il est facile de voir si on a affaire à l'une ou l'autre de ces catégories de silice en effectuant une séparation témoin de deux ou trois acides; si la silice est correcte, on doit obtenir des pics analogues à ceux de la figure 1.

La figure 7 indique le résultat d'une séparation effectuée avec de la silice préparée à partir de silicate de Na suivant L. M. Marshall et coll. (35) et en utilisant 8 ml d'acide sulfurique 0,5 N pour 8 g de silice, les autres conditions étant celles de la méthode de W. A. Bulen (8). On voit, en comparant les pics des figures 1 et 7 correspondant aux mêmes acides, que la silice d'après Marshall et coll. se prête bien à la chromatographie des acides carboxyliques, mais elle donne des pics moins aigus que la silice de Mallinckrodt de certains lots.

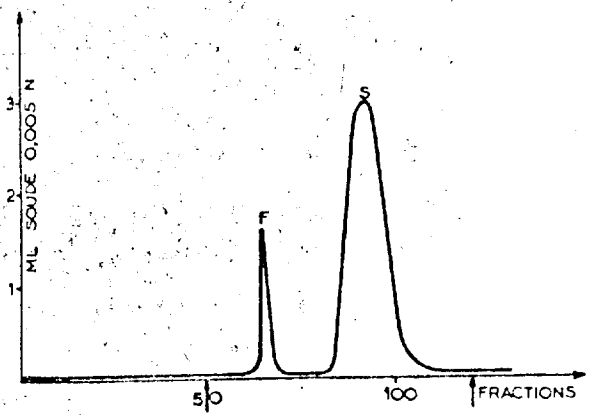

Fig. 7. — Chromatogramme des acides fumarique (F) et succinique (S) obtenu d'après la méthode de Bulen et coll. au moyen de la silice préparée suivant Marshall (34).

III. — Introduction des acides carboxyliques sur la colonne de silice.

On trouvera dans la deuxième partie de ce travail une méthode de préparation de la colonne de silice.

Pour introduire les acides carboxyliques au sommet de la colonne il existe plusieurs procédés :

1° L'échantillon des acides est dissous dans un ou deux ml d'un mélange alcool amylique tertiaire-chloroforme (26, 35), éther-alcool amylique tertiaire (37), ou butanol normal-chloroforme (36), que l'on place au sommet de la colonne.

2° L'échantillon est dissous dans 0,5 ml par exemple de phase aqueuse et on ajoute 1 g de silice; le mélange additionné de chloroforme est versé sur la silice déjà tassée dans le tube de chromatographie (8).

3° L'échantillon dissous dans un très petit volume de phase aqueuse (0,15 ml ou moins) est introduit directement avec une micropipette au sommet de la colonne de silice, à dessein soussaturée en phase aqueuse (55).

4° On fait absorber l'échantillon d'acide par un disque de papier filtre très poreux dont le diamètre est égal au diamètre intérieur du tube, la rondelle séchée est enfoncée sur le sommet de la colonne de silice; on fait passer les acides dans la colonne soit avec quelques dixaines de ml de solvant organique (55, 30), soit avec un plus petit volume encore (0,05 ml de phase aqueuse (50).

Il semble que l'introduction des acides sur la colonne avec des solvants organiques ait été abandonnée à cause des grandes différences de solubilité des acides carboxyliques dans les solvants. Pour transférer une grande quantité d'acides sur la colonne, l'emploi de silice est très commode et on obtient des résultats quantitatifs. Le procédé au disque de papier s'applique davantage à de petites quantités d'acides; il peut être avantageux quand il s'agit de rechromatographier sur silice deux acides non séparés lors d'une première chromatographie. L'introduction des acides sur la colonne dans la phase aqueuse a certains avantages : les sels d'acides volatils introduits de cette façon peuvent être hydrolysés par addition d'acide sulfurique sur la colonne même, et les pertes sont très limitées (55).

IV. — La phase mobile et l'élution.

Les mélanges de solvants.

On utilise principalement les mélanges de butanol normal (26, 35, 36, 8, 50, 46, 28) de butanol tertiaire (45), d'alcool amylique normal (15), d'alcool amylique tertiaire (35, 21) ou des alcools amylique normal et amylique tertiaire (34, 31) d'une part et de chloroforme d'autre part. La méthyl isobutyl cétone (24) et l'éther (51) ont été utilisés non mélangés à d'autre solvants.

Les acides succinique et lactique ne sont pas séparés quand on élue avec le mélange butanol normal-chloroforme; par contre le mélange butanol normal-carbure aromatique (37) et en particulier le mélange butanol normal-benzène (37, 8, 52) réalisent leur séparation. Le mélange éther-benzène réalise la séparation des acides succinique, lactique et α-cétoglutarique (30); il convient moins bien pour les acides citrique, isocitrique, tartrique et même pour l'acide malique.

L'emploi de l'alcool butylique normal est critiquable car vu la grande surface de réaction et l'acidité de la colonne, il peut se produire entre l'alcool et certains acides carboxyliques, dont principalement l'acide oxalique, une estérification (8, 45) d'autant plus importante que l'écoulement du solvant est plus lent. L'alcool butylique tertiaire et l'alcool amylique tertiaire, difficilement estérifiables dans les conditions de la colonne, ne présentent pas cet inconvénient. L'alcool butylique tertiaire a en outre l'avantage de permettre des tirages en présence d'une seule phase, donc plus faciles (45).

Pour éviter les pertes dues à l'estérification, on a aussi proposé (50) des mélanges où les cétones remplacent les alcools, et en particulier le mélange méthyl isobutyl cétone-chlorure de méthylène, le chlorure de méthylène ayant l'avantage sur le chloroforme de conduire dans ce cas à des « pics » plus aigus. Mais jusqu'à présent, les séparations réalisées au moyen de cétones ne semblent pas être aussi efficaces que celles obtenues avec les alcools : les acides succinique et lactique ne sont pas séparés non plus que les acides citrique et isocitrique.

Certains auteurs (47) n'ont pas trouvé dans les risques d'estérification une source de difficultés. Cependant, à pouvoir séparateur égal, il est préférable d'utiliser un système d'éluant qui évite l'estérification. A la suite de Ruzicka et coll., nous avons choisi le mélange butanol tertiaire-chloroforme qui n'entraîne pas des pertes par estérification et qui, contrairement à ce qu'on a pu dire (47), donne des séparations au moins aussi efficaces que le butanol normal (cf. deuxième partie)

L'élution.

Pour effectuer l'élution on peut utiliser, soit un certain nombre de mélanges de solvant introduits sur la colonne l'un après l'autre, chaque mélange se distinguant du précédent par une concentration plus élevée en constituant le plus polaire (alcool, cétone, éther, ...) on réalise ainsi une augmentation progressive mais discontinue de la polarité, soit un seul mélange dont la concentration en composé le plus polaire augmente progressivement mais en outre d'une façon continue (15, 34).

a) *Augmentation progressive mais discontinue de la polarité.*

C'est le procédé le plus utilisé (36, 21, 8, 24, 46, 47, 50, 45, 28). Il est d'application très souple, on peut faire varier à volonté le volume et la composition des mélanges et adapter la méthode à tel ou tel matériel d'analyse, à tel ou tel mélange d'acides. Mais on peut lui reprocher de nécessiter une surveillance au moment des changements de solvants lorsque ceux-ci sont effectués manuellement, afin que à l'instant où l'un des mélanges est épuisé, il n'entre pas d'air dans la colonne.

On a décrit récemment des appareils avec lesquels le changement de solvant devient automatique (1, 48), et la méthode peut être adaptée alors au travail de routine

b) *Augmentation progressive et continue de la polarité.*

Les mélanges à augmentation continue de la polarité sont obtenus dans des « chambre de mélange ». Dans l'appareil de DONALDSON K. O. et coll. (15) le composé polaire (butanol normal ou alcool amylique normal) est introduit dans le bas d'une chambre de mélange, remplie de chloroforme où les deux liquides se mélangent progressivement par suite de leur différence de densité. BOCK R. M. et coll. (2) ont décrit et étudié diverses chambres de mélange. Quand on adopte un tel dispositif, on ne peut pas équilibrer certains solvants avec la phase aqueuse (48). Bien qu'on puisse faire varier la vitesse d'augmentation de la polarité, la loi de variation de la polarité est fixée une fois pour toutes et il en découle une certaine rigidité analogue à celle qui résulte de l'utilisation des appareils à changement automatique de solvant. Le dispositif de DONALDSON K. O. et coll. (15) utilisé avec le mélange alcool amylique normal + alcool amylique tertiaire-chloroforme (34) ou avec le mélange éther-benzène (30) a permis la séparation des acides succinique, lactique et α-cétoglutarique.

Vitesse d'écoulement de l'effluent.

La vitesse d'écoulement ne doit pas être trop lente avec les solvants stérifiables; on recueille généralement 2 à 3 ml/mn: Elle dépend:

a) de la nature de la silice; *b*) de sa granulométrie; *c*) de la hauteur de la colonne de silice; *d*) du degré de tassement de la silice; *e*) de la présence de tensions interfaciales air-liquide, la présence d'air dans la colonne ralentissant l'écoulement (44); *f*) du degré d'hydratation de la silice, la vitesse d'écoulement augmentant avec la quantité de phase aqueuse par gramme de silice (19), *g*) du poids, donc de la hauteur de la colonne de liquide qui surmonte la silice; *h*) de la nature des solvants utilisés; elle diminue au fur et à mesure que croit la concentration en alcool dans les mélanges alcool-chloroforme. Avec les mélanges à base de cétones, elle est d'autant plus faible que le poids moléculaire de la cétone est plus grand (50).

On peut augmenter la vitesse d'écoulement, soit le plus souvent en exerçant une pression d'azote ou d'air comprimé sur la colonne, soit en augmentant la hauteur de la colonne de liquide qui surmonte la colonne de silice. Quand on crée la pression au moyen d'un gaz comprimé, il est bon d'avoir un manostat, manostat à échappement de gaz par exemple.

Volume des fractions recueillies.

Dans la grande majorité des cas on recueille des fractions de 2 à 5 ml.

V. — Détermination et caractérisation des acides après séparation.

En général, les acides carboxyliques sont déterminés par titrage, mais les acides polycarboxyliques peuvent aussi être déterminés fluorimétriquement.

Méthode titrimétrique.

Les acides sont titrés dans leurs fractions respectives, additionnées de quelques ml d'eau, le plus généralement par la soude diluée (0,01 N ou 0,005 N) plutôt que par la baryte (23), en présence de rouge de phénol qui donne des virages plus nets (37) que le bleu de thymol (26, 35).

Vers la fin du titrage, il est nécessaire d'agiter vigoureusement le mélange non miscible de solvant organique et d'eau, pour faire passer l'acidité non encore titrée de la phase organique dans la phase aqueuse. On peut agiter au moyen d'un jet d'air privé de CO_2 mais quand on titre dans des tubes il est plus simple et tout aussi bien d'agiter à la main en bouchant le tube avec un bouchon de caoutchouc.

Quand l'effluent est un mélange butanol tertiaire-chloroforme on peut placer les tubes après addition de quelques ml d'eau au bain de vapeur pour chasser le chloroforme dont le départ laisse un liquide d'une seule phase (45), plus faible à titrer. Nous avons trouvé que le titrage en présence des deux phases ne présentait pas de difficulté et nous n'avons jamais éliminé le chloroforme.

JOLCHINE G. (28) titre en présence de lauryl sulfonate de Na qui assure par agitation un contact intime entre les deux phases. Cependant, quand les deux phases forment une émulsion assez stable les colorations dues à l'indicateur coloré sont plus pâles et on apprécie parfois moins bien le virage. C'est pourquoi en présence d'acide malique et surtout d'acide citrique, dont les coefficients de partage sont nettement en faveur de la phase aqueuse, nous évitons la formation d'émulsion en n'agitant que légèrement (18) aussi longtemps que la phase aqueuse reste jaune (Indicateur rouge de phénol); quand la coloration passe au violet nous agitons vigoureusement; il suffit alors d'une ou deux gouttes de soude 0,005 N pour terminer le titrage.

Méthode fluorimétrique.

Il est rare que l'échantillon introduit sur la colonne ne contienne pas en même temps que les acides organiques des acides minéraux. Ces derniers sont eux aussi élués, bien qu'assez tardivement, et ils peuvent être superposés à certains acides polycarboxyliques. Ainsi l'acide nitrique peut être élué en même temps que l'acide isocitrique. Le titrage ne permet pas alors de déterminer exactement l'acidité organique. C'est en partie pourquoi, C. E. FROHMAN et J. M. ORTEN (20) ont mis au point une méthode fluorimétrique pour la détermination des acides polycarboxyliques après séparation chromatographique. Elle est basée sur la formation de dérivés fluorescents en présence de résorcinol. L'intensité de la fluorescence est mesurée dans un fluorimètre; la teinte de la fluorescence renseigne sur la nature des acides. En outre, dans le cas où les acides contiennent des atomes marqués, leurs dérivés fluorescents peuvent être extraits par l'éther dans lequel ils sont solubles, en vue de mesures de radioactivité.

Considérations quantitatives — Sensibilité.

Les recouvrements sont bons quand la silice est pure et n'adsorbe pas. Ils atteignent au moins 90 % à 95 %, et souvent les acides carboxyliques sont déterminés à moins de 5 % près. Pour l'acide oxalique élué par un solvant estérifiable, les recouvrements ne sont que de 85 à 90 %. Quand les « à blancs » sont faibles on peut déceler et estimer jusqu'à 1 ou 2 μ eq. d'acide.

Caractérisation des acides séparés.

Quand, d'une séparation à l'autre, on opère toujours dans les mêmes conditions, les résultats sont très reproductibles, et les acides individuels du certains groupes d'acides sont toujours élués dans les mêmes fractions d'effluent à quelques ml près. La place d'un pic sur le chromatogramme donne donc des présomptions sur la nature de l'acide correspondant. Mais plusieurs acides peuvent être élués dans les mêmes fractions et ne donner qu'un seul pic et un acide indéterminé peut être élué dans les mêmes fractions que l'acide généralement attendu à cet endroit. D'où nécessité de caractériser le ou les acides correspondant à chaque pic par chromatographie sur papier, par réaction spécifique, par formation de dérivés caractéristique, par l'équivalent de neutralité,...

a) *Chromatographie sur papier.*

On utilise la chromatographie à une dimension (28, 45) à deux dimensions (45, 14) ou la chromatographie circulaire (50) plus rapide que la chromatographie bidimensionnelle et même monodimensionnelle. Il est vrai que par addition aux solvants de 8 quinolinol par exemple (45) on peut rendre les taches sur le papier fluorescentes et les observer sous lumière ultra-violette avant même séchage, ce qui n'empêche d'ailleurs pas de les révéler normalement par la suite (17, 39).

b) *Réactions spécifiques.*

Formation de composés fluorescents par réaction entre les acides polycarboxyliques et le resorcinol (20) entre l'acide malique et le β-naphtol (28), l'acide citrique et le chlorure de thionyle (28). Transformation de l'acide isocitrique en lactone sous vide au bain-marie à 100° pendant 2h puis chromatographie sur papier (11); nous effectuons plus simplement la transformation de l'acide isocitrique en lactone, directement sur papier (cf. deuxième partie). Dégradation enzymatique spécifique et caractérisation des produits de dégradation, etc.

VI.—Remarques concernant certains groupes d'acides. Limites de la méthode. Comparaison de la chromatographie de partage et de la chromatographie par échange d'ions.

Acides volatils.

Il est certain que les acides volatils sont beaucoup mieux séparés et déterminés par chromatographie de partage gaz-liquide suivant la méthode de James A. T. et Martin A. J. P. (27). Il y a intérêt à les déterminer suivant cette méthode, qui est aussi légèrement plus sensible. On peut les éliminer alors de l'extrait des acides; dans ces conditions ils ne gênent pas dans la chromatographie sur silice des acides non volatils.

Acides cétoniques.

Les acides pyruvique et α-cétoglutarique, plus stables que les autres acides cétoniques, peuvent être déterminés assez facilement par chromatographie sur silice.

Acides oxalique et glycolique.

Ces acides ne sont pas séparés par le mélange butanol normal chloroforme ou avec le mélange méthyl isobutyl cétone-chlorure de méthylène (50). L'acide oxalique partiellement ionisé quitte souvent la colonne en « traînant » plus ou moins.

Acides glycolique et tricarballylique.

Ils ne sont pas séparés par le mélange butanol tertiaire-chloroforme; ils le sont avec le mélange alcool amylique normal — alcool amylique tertiaire — chloroforme (31).

Acides citrique et isocitrique.

Ils ne sont pas séparés par le mélange benzène-éther (30) ou méthyl isobutyl cétone — chlorure de méthylène (50). Ils le sont au moyen des mélanges butanol normal — chloroforme et butanol tertiaire chloroforme.

Acide quinique.

Hulme A. C. (24) n'a pas réussi à séparer cet acide d'une façon satisfaisante au moyen de la méthyl isobutyl cétone, ni avec le mélange butanol normal-chloroforme.

Acides minéraux.

Bien que tardivement, les acides minéraux finissent par être élués eux aussi. L'acide nitrique est souvent élué avec l'acide isocitrique. Les acides phosphorique et chlorhydrique peuvent gêner dans la détermination de l'acide tartrique. Ces acides minéraux sortent de la colonne sous forme de pics aplatis, irréguliers. L'acide sulfurique est élué bien après l'acide tartrique quand sa concentration dans la phase aqueuse de la silice contenant l'échantillon n'est pas trop grande (SO_4H_2N, et acides libres). En général, il commence à être élué quand le front d'hydratation arrive à mi-colonne.

Comparaison de la chromatographie des acides carboxyliques sur colonne de silice (chromatographie de partage) et sur colonne de résine anionique (chromatographie par échange d'ions.)

Les colonnes de résine possèdent l'avantage sur celles de silice d'avoir une plus grande capacité d'absorption pour les acides; avec les colonnes de résine, les acides peuvent être fixés sur une grande hauteur de colonne, alors qu'avec les colonnes de silice, l'échantillon des acides doit occuper une bande étroite au sommet de la colonne. Pour exploiter cet avantage, Palmer J. K. (41) a mis au point récemment une méthode de séparation des acides carboxyliques sur résine à anions fortement basique (Dowex 1, X-10) qu'on peut considérer comme une amélioration par rapport aux méthodes de chromatographie sur résine préexistantes. Cependant dans cette méthode, les quantités d'acides pratiquement introduites sur les colonnes de résine (0,2 m.éq) semblent être du même ordre de grandeur que celles utilisées avec les colonnes de silice. On verra d'ailleurs dans la deuxième partie de ce travail qu'on peut facilement introduire 1,0 m.éq. d'acides, et plus, sur une colonne de silice de petite taille. Quand on utilise une colonne de résine, il n'est pas nécessaire que le volume de l'échantillon introduit sur la colonne soit très faible (2 à 4 ml). Par contre avec une colonne de silice, ce volume doit être faible et, par conséquent, quand on désire chromatographier des quantités relativement grandes d'acides, la solution de l'échantillon doit être assez concentrée. Il y a là une difficulté qui peut cependant être facilement surmontée en utilisant une petite fraction de silice (8) pour transférer l'échantillon aqueux (0,5 à 1,0 ml) sur la colonne (cf. plus haut).

Dans la méthode de Palmer les acides malique, citrique et tartrique quittent la colonne de résine assez tôt, et leurs pics sont aigus; par contre, ces acides sont élués en dernier des colonnes de silice, et les pics sont généralement aplatis. Mais on verra dans la deuxième partie qu'avec la méthode que nous utilisons, on obtient de très beaux pics pour ces acides. La détermination des acides après séparation est plus facile après chromatographie sur silice, puisque, très généralement, il suffit de titrer les acides dans les diverses fractions (la présence de deux phases n'est pas un obstacle important pour la précision du titrage), alors que pour certains acides après séparation sur résine, il est nécessaire d'utiliser des procédés autres qu'un simple titrage (41).

On constate des variations entre les divers lots de résine (41) de même que l'on en constate entre les divers lots de silice. La séparation des acides est plus rapide sur colonne de résine que sur colonne de silice, et quand on est plus particulièrement intéressé par l'acidité totale, l'acide malique et l'acide citrique, il peut être intéressant d'utiliser la méthode de Palmer (41). Les colonnes de résine peuvent être régénérées alors que les colonnes de silice doivent être refaites avant chaque séparation.

En résumé, il semble que dans l'état actuel de la question, certaines méthodes de chromatographie de partage sur silice permettent malgré tout un travail plus fin que les techniques de chromatographie sur résine : plus grand nombre d'acides séparés lors d'une seule opération, séparation des acides plus efficace, détermination plus facile et plus rapide des acides après séparation. Les deux méthodes peuvent d'ailleurs se compléter; par exemple, dans un matériel biologique, séparation de la fraction « acide » de la fraction « neutre » par fixation des acides sur résine anionique fortement basique sous forme formiate, après avoir fixé la fraction « basique » sur résine à cations; élution totale des acides en vue de l'acidite totale, ou élution fractionnée en vue de la séparation de certains acides ou groupes d'acides, par un faible volume (100 ml) d'acide formique 6 N (38, 41); puis séparation plus poussée des acides sur colonne de silice, et détermination consécutive.

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STEROID HORMONE FORMATION BY THE RAT OVARY.

IV. Formation of Steroids from Acetate-1-^{14}C by Neoplastic Ovarian Tissue

By

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ABSTRACT

Ovaries were transplanted to the spleens of spayed female rats. After 18 months, ovarian neoplasms had developed. Slices of two different ovarian tumours were incubated with 100 μc acetate-1-^{14}C. Radioactive steroid hormone formation was assessed quantitatively by the reverse isotope dilution technique. Tissue from one ovarian neoplasm, considered to be a luteoma, formed radioactive progesterone and 20α-hydroxy-pregn-4-en-3-one. The formation of 3β-hydroxy-pregn-5-en-20-one, androst-4-ene-3,17-dione, testosterone, 3β-hydroxy-androst-5-en-17-one, 17-hydroxy-pregn-4-ene-3,20-dione, oestrone and 17β-oestradiol could not be established. No radioactive steroid hormone synthesis could be detected using granulosa cell tumour tissue.

There has been considerable speculation about the steroidal product of morphologically distinct types of cells within the ovary. There is, understandably, little direct information available about the hormonal steroids produced by individual types of ovarian cells, i. e., granulosa or luteal cells. We have previously reported that non-neoplastic ovarian tissue transplanted to the spleens of castrated male or spayed female rats can form *in vitro* radioactive progesterone and 20α-hydroxy-pregn-4-en-3-one from acetate-1-^{14}C (*Rice & Segaloff* 1966 *a, b*). We have also demonstrated the additional formation of radioactive oestrone and 17β-oestradiol from acetate-1-^{14}C by slices of ovarian tissue from female rats in parabiosis with castrated male rats or from female rats given exogenous gonadotrophins (*Rice & Segaloff* 1966 *c*). All of these ovarian preparations were composed of varying amounts of follicular, luteal and interstitial tissue, thus not allowing for the cellular localization of steroid hormone synthesis.

A study of functional ovarian neoplasms composed of homogeneous cell types would offer one approach to the problem of cellular localization of steroid hormone production. In the rat, neoplasms which are considered to be functional, frequently develop in ovarian tissue transplanted into the spleens of spayed animals (*Biskind & Biskind* 1949). Continuous exposure to large amounts of pituitary gonadotrophin is thought to induce neoplasia in the transplanted ovaries. Relatively large amounts of ovarian tissue of a specific cell type may be obtained by this method. Transplanted ovaries that had undergone neoplastic transformation and which predominantly represented two basically distinct types of ovarian cells, granulosa and luteal, were encountered. Steroid hormone synthesis from acetate-1-^{14}C by these ovarian tumours was studied and forms the basis for this report.

MATERIALS AND METHODS

Chemicals

The chemicals were described in previous reports (*Rice & Segaloff* 1966 *a, b, c*). 20α-Hydroxy-pregn-4-en-3-one-7α-^{3}H (1.41 mc/mg) was a gift of the Upjohn Company. The other tritium labeled steroids used as carriers were of high specific activity and were obtained commercially. All radioactive steroid carriers were rechromatographed before use. Ovine luteinizing hormone (NIH-LH-S1) was a gift of the Endocrinology Study Section of the National Institutes of Health.

Experimental design and preparation of ovarian tissue

Six to eight week old AXC female rats Line 9935, inbred and maintained in this laboratory were spayed and an ovary transplanted to the spleen of each animal. After 18 months, several animals were killed. Neoplastic ovarian tissue was present as large tumours which had not broken through the capsule of the spleen or formed adhesions. The individual ovarian tissues were removed and trimmed of splenic tissue. After weighing, the tissues were distributed among three beakers containing 100 μc (3.5 μmoles) acetate-1-^{14}C in 5 ml Krebs-Ringer bicarbonate medium, p_H 7.4. Tumour tissue from one rat (Exp. A) was cystic and soft and had the gross appearance of a granulosa cell tumour. Slices from this tumour were divided into two beakers and 100 μg of luteinizing hormone (NIH-LH-S1) was added to one of the beakers. Tumour tissue from the second rat (Exp. B) contained firm yellow-orange nodules and had the appearance of a luteoma. Slices of this tumour were incubated without added gonadotrophin. The beakers were incubated with shaking for five hours at 37°C in an atmosphere of 95% oxygen and 5% carbon dioxide. Following the incubation, the tissues were frozen and kept in the frozen state until they were extracted.

Extraction and preliminary purification

The principles of the analysis by reverse isotope dilution and the methods of extraction and preliminary purification were previously described (*Rice & Segaloff* 1966 *a, b, c*). Tracer amounts of tritium labeled progesterone, 20α-hydroxy-pregn-4-en-

3-one, 3β-hydroxy-pregn-5-en-20-one, androst-4-ene-3,17-dione, testosterone, 17-hydroxy-pregn-4-ene-3,20-dione, 3β-hydroxy-androst-5-en-17-one, and 100 μg each of non-radioactive oestrone and oestradiol were added to each incubation vessel before extraction to allow for calculation of losses occurring during isolation of the radioactive steroids formed from acetate-1-^{14}C during the incubation. Tracer amounts of tritium labeled carriers were used to allow for the measurement of μg amounts of ultraviolet absorbing steroids, if they were encountered during the initial chromatography on paper. No attempt was made to measure μg amounts of endogenously formed steroids that would not absorb ultraviolet light emitted by a short-wave ultraviolet lamp (253 mμ).

General chromatographic procedure and measurement of radioactivity

The general procedure for isolation of carrier steroids by paper and thin-layer chromatography has been described (*Rice & Segaloff* 1966 *a, b, c*). The two oestrogens were separated from the less polar neutral steroid carriers by paper chromatography for 20 hours in the ligroin/propylene glycol system. Oestrone and 17β-oestradiol were then purified by additional thin-layer chromatography (*Rice & Segaloff* 1966 *c*). The less polar steroids on the chromatograms or in the runoff were seprated as previously described by chromatographing them for shorter periods of time in the ligroin/propylene glycol system and by thin-layer chromatography and derivative formation in certain instances (*Rice & Segaloff* 1966 *a, b*). In the ligroin/propylene glycol system 20α-hydroxy-pregn-4-en-3-one is not well separated from 3β-hydroxy-androst-5-en-17-one. These two steroids were purified in this study after formation of the acetate derivatives. 20α-Acetoxy-pregn-4-en-3-one was then separated from 3β-acetoxy-androst-5-en-17-one by two dimensional thin-layer chromatography in ligroin-ethyl acetate (5:2) and benzene-ethyl acetate (4:1). Recoveries of all steroid carriers were usually over 50 %. Once it was determined by paper chromatography that no endogenous ultraviolet absorbing steroids could be measured, 100 μg of non-radioactive steroid were added for each tritium labeled carrier before subsequent separation of the carriers by thin-layer chromatography.

The radioactivity associated with each carrier steroid was measured with a liquid scintillation spectrometer. Counting efficiency averaged 60 % for carbon-14 and 30 % for tritium. ^{14}C and tritium were counted simultaneously by the method of *Okita et al* (1957). All ^{14}C measurements were corrected to 100 % counting efficiency, adjusted for the weight of tissue and recovery of tritium labeled or non-radioactive carrier steroid, and recorded as disintegration per minute (dpm) per g tissue.

Additional methodology

The procedure for thin-layer chromatography, location and estimation of carrier steroids, elution, acetylation, and detection of radioactivity on paper chromatograms have been described (*Rice & Segaloff* 1966 *a, b, c*).

Criteria of radiochemical purity

Our criteria for radiochemical homogeneity require close agreement of the specific activities of crystals and mother liquor residue from the final crystallization. Agreement in the ratios of the radioactivity measurements of ^{14}C and tritium of successive crystals and mother liquor residues yields additional evidence for the identity of the radioactive steroid formed from acetate-1-^{14}C, the tritium labeled carrier steroid, and the non-radioactive steroid used for the crystallizations.

RESULTS

Histology

Representative sections of the neoplastic ovarian tissues are shown in Figs. 1 and 2. One tissue (Fig. 1) was predominantly composed of granulosa cells and was considered to represent a granulosa cell tumour although study of additional areas of the tumour revealed small amounts of luteal tissue. The second tumour (Fig. 2) was a luteoma predominantly. Small groups of granulosa cells were scattered throughout this ovarian tumour. Both tumours were similar to those tumours described by *Biskind & Biskind* (1949).

Steroids formed from endogenous precursors

No UV absorbing zones were detected during the initial paper chromatography of the extracts of either tumour.

Steroids formed from acetate-1-^{14}C

(A) *Granulosa cell tumour.* No ^{14}C containing zones of radioactivity were detected after scanning the initial paper chromatograms with a 4 pi gas flow chromatogram scanner. In order to improve the chances of detection, the carrier steroids from the control incubation were pooled with the carrier steroids from the gonadotrophin incubation (NIH-LH-S1) after paper chromatography and before subsequent thin-layer chromatography. Only traces (< 300 dpm/g) of ^{14}C were present with any of the steroids after final chromatographic separation of the carriers. Of particular interest, oestrone and 17β-oestradiol were associated with the smallest amounts of radioactivity – 26 and 27 dpm/g of tissue respectively. We could not establish the formation of any radioactive steroid hormones from acetate-1-^{14}C by this granulosa cell tumour.

(B) *Luteoma.* Two radioactive zones, corresponding in mobility to progesterone and 20α-hydroxy-pregn-4-en-3-one, were detected on the initial paper chromatograms. The radioactivity associated with progesterone was treated with acetic anhydride and pyridine to remove acetylable impurities and chromatographed on thin-layer silica gel plates as described previously (*Rice & Segaloff* 1966 *a*). 20α-Acetoxy-pregn-4-en-3-one was separated from 3β-acetoxy-androst-5-en-17-one after acetylation of the free compounds and subsequent thin-layer chromatography. Additional non-radioactive progesterone and 20α-acetoxy-pregn-4-en-3-one were added and each steroid sample was crystallized to constant specific activity (Table 1). The data of Table 1 indicate that both steroids were nearly radiochemically homogeneous as isolated following these chromatographic procedures. Only traces of ^{14}C were associated with any of the other carrier steroids from this experiment despite satisfactory recoveries of the carriers. We conclude that, if these other steroids were formed, they were formed in extremely small amounts from acetate-1-^{14}C.

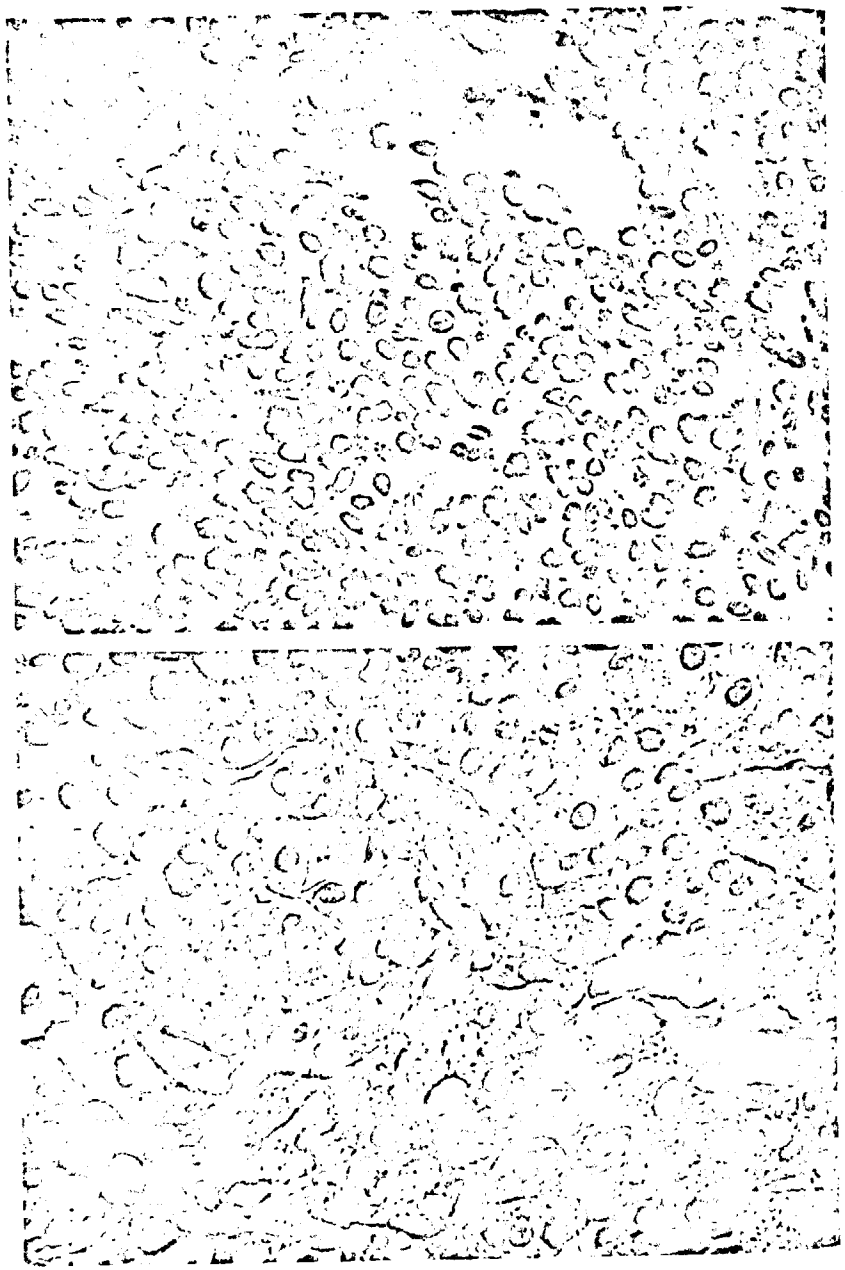

Fig. 1. Granulosa cell tumour removed from the spleen of a spayed female rat 18 months after transplantation of normal ovary. × 800.

Fig. 2. Luteoma removed from the spleen of a spayed female rat 18 months after transplantation of normal ovary. × 800.

Table 1.
Radiochemical purification of steroids formed from acetate-1-^{14}C by rat ovary tumour (luteoma).

Compound		Progesterone	20α-Hydroxy-pregn-4-en-3-one[a]
Radioactivity (dpm)		31 800	41 800
Steroid carrier (mg)		10.5	12.3
Calculated specific activity (dpm/mg[b])		3030	3400
Specific activity (dpm/mg)			
1st	Crystals	2940	3300
	Mother liquor	4260	3700
2nd	Crystals	2900	3270
	Mother liquor	3000	3380
Ratio $\frac{^{14}C\ cpm}{^{3}H\ cpm}$			
1st	Crystals	.306	1.02
	Mother liquor	.275	1.03
2nd	Crystals	.312	1.01
	Mother liquor	.300	1.02

a) Crystallized as 20α-acetoxy-pregn-4-en-3-one.
b) After addition of carrier.

DISCUSSION

In this limited study, tissue consisting predominantly of neoplastic granulosa cell elements (Fig. 1) did not appear to form radioactive steroids from acetate-1-^{14}C, while the luteoma (Fig. 2) did synthesize progestational steroids (Table 2). Progesterone and 20α-hydroxy-pregn-4-en-3-one were the only identified radioactive steroids formed *in vitro* from acetate-1-^{14}C by this luteoma. These findings are in keeping with the traditional view that the luteal cell is a source of progestational steroids produced within the ovary.

Our results with this granuloma cell tumour of the rat's ovary are reminiscent of the findings of *Ryan & Short* (1966) with granulosa cells isolated from the equine follicle. They were similarly unable to demonstrate steroid hormone synthesis from acetate-1-^{14}C by a preparation consisting almost entirely of granulosa cell elements. They proposed that a combination of granulosa and luteal cell elements was needed for total follicular steroid biosynthesis from acetate-1-^{14}C. Aromatization of radioactive testosterone to oestrone and oestradiol has been reported in studies with a human feminizing granulosa cell tumour of the ovary (*Marsh et al.* 1962) as well as a granuloma cell preparation of the equine follicle (*Ryan & Short* 1965). In a recent study, *Ryan & Petro*

Table 2.
Steroid hormone formation *in vitro* by transplanted rat ovary[a].

Exp. No.	Tumour	Tissue g	Gonadotrophin NIH-LH-S1 *in vitro* (μg)	Acetate-1-^{14}C incorporation into steroid (dpm/g tissue)[b]		
				Progesterone	20α-OH-pregn-4-en-3-one	Others[c]
A-1	Granulosa cell	1.49	none	N. D.[d]	N. D.	N. D.
A-2	Granulosa cell	1.31	100	N. D.	N. D.	N. D.
B-1	Luteoma	1.48	none	38 200	55 000	N. D.

a) Incubated 5 h in 5 ml Krebs-Ringer bicarbonate medium, containing 100 μc (4.5 μmoles) acetate-1-^{14}C, p_H 7.4, 95 % O_2 – 5 % CO_2.
b) Acetate-1-^{14}C incorporation based on measured radioactivity as eluted from final chromatogram and adjusted for recovery of carrier steroid.
c) See text for complete list of steroid carriers.
d) N. D. = not detectable; carrier steroids from A-1 and A-2 were pooled after initial paper chromatography to enhance the detection of radioactive steroid synthesis from acetate-1-^{14}C.

(1966) incubated granulosa and thecal elements of human follicles with labeled progesterone and pregnenolone. In their study with these separated elements of the human follicle, steroid conversions were demonstrated but oestrogen formation was relatively low (*Ryan & Petro* 1966). This results contrasts with the earlier studies of *Smith & Ryan* (1961) in which oestrogens were the major radioactive steroids formed *in vitro* from acetate by minced human ovarian follicles, but was similar to the results they obtained when they used radioactive progesterone as a precursor.

These results obtained with neoplastic rat ovarian tissue should be interpreted in the light of the limited knowledge of ovarian steroid synthesis by the rat's ovary. It appears from *in vivo* and *in vitro* studies that progestins are the predominant steroids formed by the rat's ovary at all times during its normal reproductive life (*Rice & Segaloff* 1966 *a, b, c*; *Wiest* 1959; *Eto et al.* 1962; *Telegdy & Endröczi* 1963). The formation of radioactive oestrogens from acetate-1-^{14}C has only recently been demonstrated in the rat's ovary (*Rice & Segaloff* 1966 *c*). In these highly stimulated ovarian preparations from rats in parabiosis with castrated male partners or from rats given exogenous gonadotrophins, acetate incorporation into radioactive oestrogens was still only a small fraction of that found in the progestins (*Rice & Segaloff* 1966 *c*).

Studies of neoplastic tissue are not, of course, necessarily representative of normal tissue and may on occasion, be misleading. In addition, accurate morphologic classification of ovarian neoplasms is difficult at best. However, this study has demonstrated that neoplastic ovarian tissue with the predominant morphological characteristics of a luteoma is capable of forming *in vitro* progestational steroids from acetate-1-^{14}C. No discernible steroid synthesis from acetate-1-^{14}C was detected in a granulosa cell tumour incubated at the same time. It would appear from this study, and others, that additional information is needed to clarify the steroidogenic capabilities of the granulosa cell.

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Milk Fat Synthesis from Acetate in Mammary Gland of the Cow. (23059)

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Previous publications from our laboratory have been concerned with the roles of short chain fatty acids, bicarbonate and glucose as precursors of milk-constituents(1-9). These have been studied by intravenous injection of various metabolites appropriately labeled with C^{14}. Acetate is the principal metabolite derived from fermentation of carbohydrates in the rumen(10), and we have shown that it is a precursor of all milk-constituents, as well as being extensively catabolised to meet energy requirements. As might be expected, plasma acetate is a potent precursor of the fatty acid moiety of milk fat, but acetate carbon is also utilized in synthesis of the glycerol moiety. In view of the large amount of acetate available to cow's tissues, this latter synthesis is probably an important source of glycerol. The evidence indicates that it takes place via the tricarboxylic acid cycle as well as via fixation of carbon dioxide derived from acetate oxidation(8,9).

The work of Popjak, Folley *et al.*(11,12) with perfused udders has shown that a considerable part of milk fat synthesis from small molecules takes place in the mammary gland itself. In this experiment 2-C^{14} labeled acetate was injected into the cistern of one quarter of the mammary gland of an intact cow, and radioactivities of milk fat components from that quarter were compared with those from the other 3 quarters and with those from a similarly labeled acetate injection made intravenously.

Methods. The technic for conducting tracer experiments with intact cows has been described by Kleiber(1). Following injection of labeled acetate, continuous samples of respired carbon dioxide were taken for the first 3 hours and then for short periods at intervals up to 35 hours. The cow was milked at 3, 10, 22, and 35 hours after injection. Details of injections and characteristics of the cows are tabulated in Table I. The fats were separated from milk samples and 4 crude fractions prepared from each. These were the glycerol; water-soluble steam-volatile fatty acids, water-insoluble steam-volatile fatty acids, and non-volatile fatty acids. Details of separation are described by Rogers (9). A sample of each fraction was combusted and the resultant carbon dioxide was trapped as barium carbonate. This was planchetted and counted at infinite thickness in a flow-gas counter. Udder injection of acetate was made into the right front quarter by passing a large blunt needle up the teat canal and depositing the acetate in the milk cistern.

TABLE I. Details of Animals Used and Isotope Injected.

	Udder inj. exp.	Intrav. inj. exp.
Wt of cow	478.5	470
Milk production, kg/day	7.4	7.6
Fat, %	4.8	5.7
Isotope inj.	1.20 mc $C^{14}H_3COOH$	3.86 mc $C^{14}H_3COOH$

TABLE II. Integrated Radioactivities of Milk Fat Constituents.

	$\sum_0^\infty \lambda_s \Delta t$				$\int_0^\infty \rho_s \, dt$
	Glycerol	Sol. VFA	Insol. VFA	NVFA	
Udder injection RFQ	30.51	249.6	431.04	171.78	3.96
" " 3Q	16.07	43.3	61.75	15.44	
Intrav. injection	27.9	79.7	83.1	16.8	62.78

This quarter was subsequently milked out separately from the others, and the data referring to this milk are henceforth indicated by the abbreviation RFQ. A similar abbreviation for the other 3 quarters is 3Q, and the intravenous injection data are indicated by the abbreviation i.v.

Results. The results are summarized in Table II and Fig. 1. Radioactivity of each sample was determined as "Standardized Specific Activity λ,"(2) of which the units are μc/g atom carbon : μc injected/kg body weight. By dividing specific activity by administered dosage, it is possible to compare results from experiments in which different dosages were injected into animals of different weights. As metabolic processes take place at different rates, the specific activity of a milk-constituent is not necessarily a guide to the extent to which it is synthesized from injected metabolite. The best indication of the potency of an injected metabolite as pre-

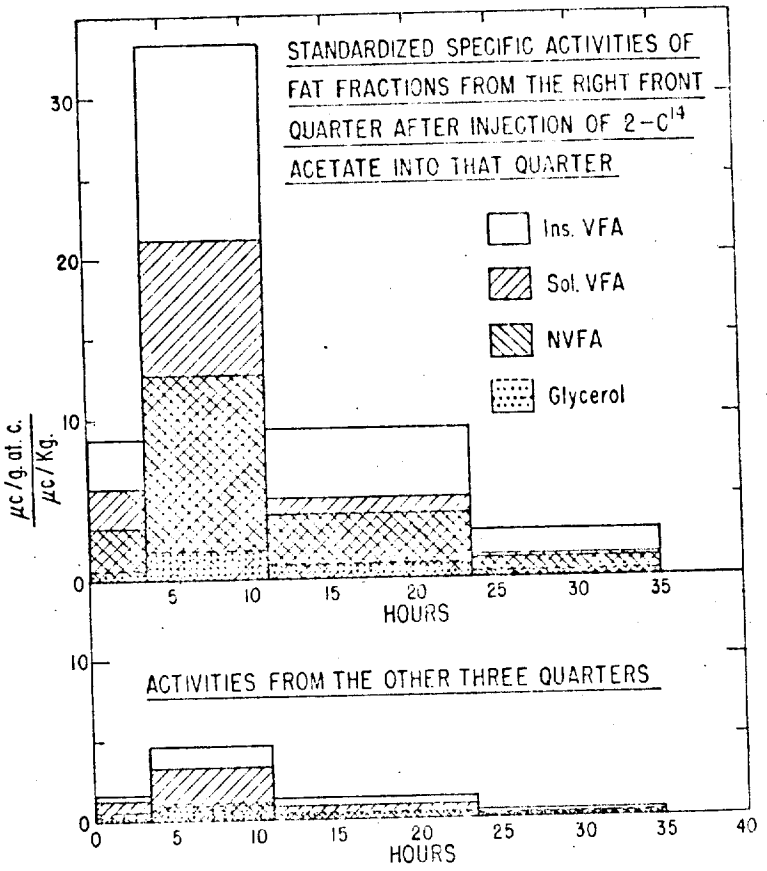

FIG. 1.

cursor of a milk-constituent is given by the time integral of specific activity of that constituent. This is easily determined since specific activity of a milk-constituent from any milk sample is the average specific activity of that constituent which has been secreted since the previous milking. Therefore, each average radioactivity can be multiplied by the number of hours in its milking period to give the integral for that period, and the sum of these will be the integral for the entire period $\sum_0^{35} \lambda_s \Delta t$. The time integral of the respired carbon dioxide activity is designated $\int_0^{35} \rho_s \, dt$.

The graphs in Fig. 1 illustrate the remarkably different activities of fat fractions from the RFQ and those from the 3Q. The fact that there is a difference shows that at least some of each fraction is synthesized in the mammary gland itself. The magnitudes of the differences are illuminating. Glycerol is only twice as radioactive from the RFQ as from the 3Q, whereas the 3 fatty acid fractions are more radioactive by factors of 6, 7, and 11 for soluble-volatile, insoluble-volatile and non-volatile fatty acids, respectively. In the RFQ the glycerol has much lower radioactivity than fatty acid fractions associated with it. In the 3Q, however, the relative difference is smaller and the ratio of radioactivities of glycerol and fatty acids is of a similar order to that seen previously where the labeled acetate was injected intravenously. From these 2 observations one can infer that there is less glycerol synthesis from acetate in the mammary gland than there is fatty acid synthesis. It is also apparent that mammary tissue cells are extremely active in synthesis of fatty acids of all chain lengths.

One of the most striking results from the udder injection experiment is that labeled acetate seemed to stay in the mammary gland and did not distribute throughout the body of the cow. This is clearly shown by the very small time integral of radioactivity of respired carbon dioxide, which is only 6.4% of that following a similar intravenous injection. It might be concluded from this that certainly less than 10% of injected acetate found its way into the circulation despite the vascularity of the udder. Similarly, the enormously greater radioactivity of milk fat constituents in the RFQ than in the 3Q and the persistence of the difference through 35 hours suggest that diffusion through the extravascular fluid is also limited. This apparent immobilization of injected acetate is not at all consistent with other observations concerning diffusion of small molecules in fluids of the body. An explanation is suggested by some other observations. As was previously reported(9), the most radioactive fat was recovered from the second milk sample after intravenous injection of a labeled precursor. The peak radioactivities of lactose and other milk constituents were seen in the first milk sample(2-8). The same phenomenon was observed even where labeled acetate was injected into the mammary gland, showing that the delay is not due to slow transport of fat to the udder from other synthetic sites. The work of Popjak, Folley *et al.*(11,12) with surviving slices of mammary tissue shows that actual synthesis of fatty acids from acetate is rapid. Furthermore, delay of peak radioactivity is observed in the glycerol moiety of fat as well, but peak of radioactivity in the lactose(8,13) is seen in the first milk sample in all trials, including this udder experiment. Since glycerol and lactose syntheses from acetate have common pathways, it is evident that the delay is not one of synthesis. Therefore, we have additional evidence for a previous suggestion(9) that the fat from the second milk sample is always more radioactive than the first because of a delay in actual secretion of fat.

If it is assumed, therefore, that injected acetate is rapidly utilized by cells adjacent to the milk cistern for fat synthesis and that this fat is only secreted several hours later, the apparent immobility of injected acetate seems less paradoxical. Continuance of the difference of the radioactivities of fat fractions between RFQ and 3Q even after 35 hours is understandable if the mammary gland is still secreting fat which was actually synthesized very shortly after injection of labeled acetate into the RFQ. The obviously small amount of injected acetate that diffused away from the RFQ can be accounted for in two ways. First, the rate of its incorporation

into fat molecules may have been much faster than the possible passive diffusion of acetate through the extravascular fluid. Second, the rate of acetate utilization for biosynthesis and catabolism in cells adjacent to the milk cistern might be so great that there is a steep diffusion gradient down towards that area, so that the number of acetate molecules moving in the opposite direction is small.

Summary. 1. 2-C^{14} labeled acetate was injected into the milk cistern of the right front quarter of a lactating cow. This quarter was subsequently milked separately from the other 3 quarters and radioactivities of milk fat constituents from each were determined. 2. The data confirm that there is a delay of several hours between synthesis and secretion of milk fat. 3. A surprisingly small amount of the injected acetate diffused into the other 3 quarters or into the rest of the body. This can be accounted for by its rapid utilization for milk synthesis near the site of injection. 4. Fatty acids of all chain lengths and glycerol seem to be synthesized from acetate in the mammary gland itself. The glycerol synthesis, however, is on a smaller scale.

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The utilization of acetic, propionic and butyric acids by growing heifers

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Differing efficiencies of utilization of acetic, propionic and butyric acids in both fasting and fattening sheep have been demonstrated by Blaxter and his colleagues (Armstrong & Blaxter, 1957*a*, *b*; Armstrong, Blaxter & Graham, 1957). In fasting sheep, when the acids were given singly as the sole source of energy, the heat increment with acetic acid (41% of the energy of the metabolized acid) was much higher than that with propionic and n-butyric acids (13 and 16%, respectively). With a mixture (3:2) of propionic and n-butyric acids the heat increment was reduced to 9%, and the addition of acetic acid to this mixture up to a molar proportion of 90% gave only a small increase in heat increment, up to 15%. In fattening sheep, the efficiency of utilization of acetic acid (heat increment 67·1%) was much less than that of propionic and n-butyric acids (43·7 and 38·1%, respectively).

In young growing animals, protein deposition may be of equal or greater importance than fat deposition and, because of the high water content of muscle, it may contribute more than fat to any gain in body-weight, but there is no information on the relative efficiencies of utilization of the fatty acids for protein deposition.

This paper describes two experiments in which the effects of intraruminal infusions of acetic, propionic and butyric acids on nitrogen retention and on the gain in body-weight have been determined. Information was also obtained on the effects of the infusions on the digestibility of the dry matter of the diet and the intake of hay when it was offered *ad lib.*, on the total concentration and relative proportions of the volatile fatty acids in the rumen and on the concentrations of total volatile fatty acids, glucose and ketone bodies in the blood plasma. A preliminary report of the results of the first experiment has been published (Rook, Balch & Campling, 1960).

EXPERIMENTAL

Animals and their management. Four Friesian heifers with large permanent rumen fistulas were used in both experiments: their mean age was 22 months at the beginning of the first experiment and 26 months at the beginning of the second. They were housed in a metabolism house (Balch, Johnson & Machin, 1962).

Experimental design. The order of the treatments, details of which are given in Table 1, followed a Latin square design balanced with respect to residual effects in

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both experiments. The allocation of animals to standings and to treatment sequences was random in both experiments.

Foods. Over the first 25 days of the 35 days in each treatment period in Expt 1 and throughout Expt 2 the daily basal diet consisted of 5·4 kg hay (ryegrass–clover hay in Expt 1; ryegrass hay in Expt 2), 1·1 kg dairy concentrate cubes (barley 17, maize 20, wheat bran 20, decorticated groundnut meal 15, copra cake 10, palm-kernel cake 5, molasses 10, dicalcium phosphate 1, calcium carbonate 1, and salt 1%, with 5×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D added per ton) and 227 g decorticated groundnut cake. Over days 26–35 in Expt 1, the quantities of dairy concentrate cubes and of decorticated groundnut cake offered were as above, but the amount of hay offered was ½–1 kg in excess of the daily consumption. The daily ration was given in two equal feeds at approximately 12 h intervals. Water was available at all times.

Treatments. Water and the diluted acids (see Table 1) were given as continuous intraruminal infusions (see Rook & Balch, 1961) throughout each experiment. From the first introduction of an acid the daily amount was increased gradually from about one-quarter of the amount shown in Table 1 on the 1st day to the full amount on the 4th day.

Table 1. *Details of experimental design in two experiments with a group of four growing Friesian heifers*

Expt no.	Details of Latin square	Duration of each treatment (days)	Treatment: Infusion given daily into reticulo-rumen	Treatment: Calories supplied by infusion (kcal/day)
1	Balanced* 4×4	35	(*a*) 10 gal water	—
			(*b*) 954 ml acetic acid + 10 gal water	3500
			(*c*) 706 ml propionic acid + 10 gal water	3500
			(*d*) 610 ml butyric acid + 10 gal water	3500
2	Balanced* 4×4	35	(*a*) 15 gal water	—
			(*b*) 1500 ml acetic acid + 15 gal water	5500
			(*c*) 1110 ml propionic acid + 15 gal water	5500
			(*d*) 954 ml butyric acid + 15 gal water	5500

* See p. 399.

Experimental details. In each period of Expt 1 there was an introductory period of 11 days. On day 11, animals were harnessed for the separate collection of faeces and urine (Balch, Bartlett & Johnson, 1951) and quantitative collections were made over days 12–23 inclusive. On day 25, samples of rumen liquor were taken at hourly intervals and samples of jugular-vein blood every 3 h. Over days 26–35, measurements of the voluntary intake of hay, when it was offered *ad lib.*, were made. Animals were weighed after the temporary removal of the contents of the reticulo-rumen at 12.00 h on days 4 and 25 in each period.

In Expt 2, the introductory feeding period extended from day 1 to day 24 in each period and the quantitative collection of faeces was made over days 25–36 inclusive. Measurements of body-weight after the temporary removal of the contents of the reticulo-rumen were made at 12.00 h on days 1 and 2 of each period and on the

2 days succeeding the final period, during which the animals were maintained on their previous experimental diet.

Sampling and methods of analysis. Large representative samples of foods, faeces, urine and 'overflow' (spillage of faeces and urine) were taken. The urine and overflow were collected in acid and a subsample of faeces was stored under acid, for nitrogen determination. A subsample of the faeces and the samples of the foods were dried at 100° and milled before analysis.

N was determined by the Kjeldahl method. Samples of rumen liquor were filtered through muslin gauze and then centrifuged for 40 min at 2500 *g*. The centrifugate was analysed for total volatile fatty acids by the method of Annison (1954) and every fourth sample for the individual fatty acids by gas chromatography by the method of James & Martin (1952). Samples of blood plasma were analysed for total volatile fatty acids (Scarisbrick, 1952), glucose (Somogyi, 1952), acetone plus acetoacetic acid, and β-hydroxybutyric acid (Reid, 1960).

RESULTS

Nitrogen retention. The mean retention of N during the control periods was positive in both experiments: retention was slightly higher in Expt 2 (18·0 g/day) than in Expt 1 (14·6 g/day) (Table 2), probably reflecting the higher digestibility of the dry matter of the diet in Expt 2. In each experiment, all the supplements gave an increase in N retention but the differences were not significant ($P > 0.05$). The increase in N retention was, however, greatest with the acetic acid supplement (7·4 g/day) and least with the propionic acid supplement (3·6 g/day) in Expt 1, but all the supplements gave similar responses, varying only from 3·1 to 4·1 g/day, in Expt 2.

Table 2. *Mean values with their standard errors for retention of N, gain in empty-body weight, digestibility of the dry matter of the food and intake of hay offered* ad lib. *in four growing heifers given a daily intraruminal infusion of water or of acetic, propionic or butyric acid*

Expt no.	Infusion*	N retention (g/day)	Change in empty-body weight† (kg/day)	Dry-matter digestibility (%)	*Ad lib.* intake of hay‡ (kg/day)
1	Water	14·6	−0·11	56·2	7·5
	Acetic acid	22·0	+0·27	56·2	6·5
	Propionic acid	18·2	+0·17	57·2	7·0
	Butyric acid	19·8	+0·34	56·9	7·1
	S.E. of differences between two means	±3·24	±0·175	±0·97	±0·25
2	Water	18·0	+0·10	69·8	—
	Acetic acid	21·1	+0·61	66·5	—
	Propionic acid	21·9	+0·64	68·6	—
	Butyric acid	22·10	+0·77	70·4	—
	SE of differences between two means	±3·58	±0·088	±0·63	—

* For details of infusion see Table 1.
† For method of calculating empty-body weight see p. 402.
‡ N retention, change in empty-body weight and dry-matter digestibility were determined with intakes of hay equal for all treatments.

Empty-body weight gain. Empty-body weight was determined by subtracting the weight of total gut contents from the live weight. Total gut contents were calculated on the assumption that the amount in the reticulo-rumen made up 73% of that in the total gut (Mäkelä, 1956). On average, there was a small loss (−0·11 kg/day) in empty-body weight in animals during control feeding in Expt 1 and a small gain (+0·10 kg/day) in Expt 2 (Table 2). All the supplements gave an increase in empty-body weight gain, with a mean value of 0·37 kg/day in Expt 1 and 0·56 kg/day in Expt 2: the effect was not significant in Expt 1 ($P > 0.05$) but highly significant ($P < 0.01$) in Expt 2. Differences in the responses to the individual acids were not significant ($P > 0.05$) but the highest gains were obtained with the butyric acid supplement in both experiments.

Table 3. *Mean values of acetic, propionic and butyric acid for fat deposition and calorie retention in four growing heifers, as calculated from nitrogen retention and empty-body weight gain*

Expt no.	Infusion	Empty-body weight gain (g/day)	N retention (g/day)	Gain of fat-free* material (g/day)	Fat deposition (g/day)	Calculated† calorific value of gain in empty-body weight (kcal/day)
1	Acetic acid	377	7·4	211	166	1802
	Propionic acid	281	3·6	103	178	1787
	Butyric acid	450	5·2	148	302	3003
2	Acetic acid	477	3·1	88	389	3743
	Propionic acid	554	3·9	111	443	4280
	Butyric acid	663	4·1	117	546	5246

* Calculated from N retention, with factors given by Blaxter (1962).
† From the factors of Blaxter & Rook (1953).

Using the factors given by Blaxter (1962) for the calculation from N retention of fat-free material deposited in the body, estimates have been made of fat deposition and the results are given in Table 3. From these figures the calorific value of the gain in empty-body weight has been estimated, with the factors of Blaxter & Rook (1953), and the efficiency of utilization of the energy of the infused acids calculated; no allowance was made for changes in the digestibility of the diet. These estimates of the efficiency of utilization of the energy of the infused acids were much higher than those obtained in the calorimetric experiments of Armstrong & Blaxter (1957*b*), indicating the considerable errors that are involved in the many assumptions made: but in comparative terms the results of the two experiments described here are consistent in indicating a much higher efficiency of utilization of the energy of butyric acid than of propionic or acetic acid.

Effects of the infusion of the acids on conditions within the reticulo-rumen. The mean values for pH, the concentration of the total volatile fatty acids and the molar proportions of the individual acids in the rumen liquor in Expt 1 are given in Table 4. The intraruminal infusion of acetic acid caused a significant ($P < 0.05$) decrease, of 0·32 units, in rumen pH but the infusions of propionic and butyric acid were without

effect. The concentration of total volatile fatty acids in rumen liquor was increased with all three of the acids ($P < 0.01$), the increase noted being roughly proportional to g-equiv. of acid added: at the same time, there was an increase in the molar proportion of the acid being infused with a proportional decrease in the other acids. There was no evidence that the proportions among the acids other than the infused acid were changed, with the possible exception of a slight increase in valeric acid when propionic or butyric acid was being infused.

Table 4. *Expt* 1. *Mean values with their standard errors for pH, concentration of total volatile fatty acids and molar proportion of the individual acids in the rumen liquor of four heifers receiving intraruminal infusions of water or of a solution of acetic, propionic or butyric acid*

Infusion	pH	Total volatile fatty acids (m-equiv./100 g)	Molar proportions of individual fatty acids in rumen liquor: Acetic acid	Propionic acid	Butyric acid	Valeric acid
Water	6·35	8·66	69·1	18·2	9·8	2·8
Acetic acid	6·03	11·18	78·0	13·1	6·8	2·2
Propionic acid	6·37	10·00	52·6	36·3	8·3	2·9
Butyric acid	6·31	9·42	56·8	16·2	24·1	3·0
SE of differences between two means	±0·077	±0·388	±1·53	±1·28	±0·95	±0·22

Table 5. *Expt* 1. *Mean values with their standard errors for concentrations of sugar, total volatile fatty acids, acetone plus acetoacetic acid, and β-hydroxybutyric acid in the plasma of peripheral blood of four growing heifers receiving intraruminal infusions of water or of a solution of acetic, propionic or butyric acid*

Infusion	Sugar (mg/100 ml)	Total volatile fatty acids (m-equiv./l.)	Acetone + acetoacetic acid (mg/100 ml)	β-hydroxybutyric acid (mg/100 ml)
Water	71·8	0·36	0·18	2·09
Acetic acid	74·8	0·91	0·18	2·44
Propionic acid	73·3	0·34	0·21	1·52
Butyric acid	71·3	0·36	0·31	6·73
SE of differences between two means	±1·87	±0·075	±0·023	±0·491

Effects of the infusion of the acids on the digestibility of the dry matter of the diet and on the voluntary intake of hay (*Expt* 1). The digestibility of the dry matter of the diet was unaffected in Expt 1, but at the higher level of acid infusion in Expt 2 the infusion of acetic acid was associated with a significant ($P < 0.05$) reduction in dry-matter digestibility (Table 2). The *ad lib.* intake of hay was significantly ($P < 0.05$) depressed only by the infusion of acetic acid in Expt 1. Measurements of the *ad lib.* intake of hay were not made in Expt 2. Balch & Campling (1962) have discussed these results in relation to the chemostatic theory of the regulation of food intake by ruminants.

Effects of infusion of the acids on the composition of the blood plasma (*Expt* 1). No significant effect on the concentration of glucose in the blood plasma was observed (Table 5). The infusion of acetic acid caused a highly significant ($P < 0.01$) increase

in the total volatile fatty acid concentration of the plasma but the other acids were without effect. The infusion of butyric acid produced a very highly significant increase ($P < 0.001$) in the β-hydroxybutyric acid content of blood plasma and a proportionately smaller, but still highly significant ($P < 0.01$), increase in acetone plus acetoacetic acid content. A small increase in β-hydroxybutyric acid content with the infusion of acetic acid and a small decrease with the infusion of propionic acid were also obtained, but these effects were not significant ($P > 0.05$). These observations are consistent with present knowledge of the metabolism of the acids, and do not indicate any abnormality resulting from infusion of the acids.

DISCUSSION

The technique of continuously infusing dilute aqueous solutions of the volatile fatty acids into the rumen to study their metabolism is open to the criticism that abnormal physiological conditions might be produced within the rumen, and the digestion of the basal diet and the absorption of the end-products of digestion modified. The reductions in the voluntary intake of hay and in the digestibility of the dry matter of the diet during the infusion of acetic acid are indicative of such effects. These effects were, however, slight and there was no suggestion that the production of acids within the rumen was altered. No abnormalities due to the infusion of propionic or butyric acids were noted. Because of the restriction of the amounts of acids infused to minimize possible harmful effects, however, the responses to the infused acids in N retention in both experiments and the gain in empty-body weight in the first experiment did not achieve statistical significance.

The two experiments were consistent in indicating a higher efficiency of utilization of the energy of butyric acid than of propionic or acetic acid, as judged from the N retention and estimated fat storage: in both experiments energy was stored mainly as fat. The more precise calorimetric experiments of Armstrong & Blaxter (1957*b*), however, have indicated that the efficiency of utilization of both butyric and propionic acids for lipogenesis is considerably higher than that of acetic acid, and clearly little reliance can be placed on the information presented here concerning the relative efficiency of the utilization of the energy of the acids. In terms of N retention, however, there was a trend towards a higher response to the infusion of acetic than of butyric or propionic acid in Expt 1, and differences in the ability of the fatty acids to promote N retention may exist. It is evident that experiments should be made under more critical conditions with young animals having a greater propensity for growth than the animals used in the experiments described here.

As an outcome of the observations of Armstrong & Blaxter (1957*b*) on the relative inefficiency of the utilization of acetic acid in lipogenesis, interest has centred on the possibility of altering the efficiency of food conversion in cattle by modifying the end-products of rumen fermentation. For example, altering the physical condition of a diet, by fine-grinding of the roughage and cooking of the concentrates, and the addition to the diet of cod-liver oil or specific unsaturated fatty acids have been shown (see Shaw, 1961) to favour the production in the rumen of propionic acid at the expense

of acetic acid, and Shaw, Ensor, Tellechea & Lee (1960) observed an increased rate of weight gain and efficiency of food conversion by steers in response to the grinding of the hay and cooking of the concentrates of a diet, which they attributed largely to a changed pattern of rumen fermentation. Our limited observations do not indicate any marked difference in the efficiency with which acetic and propionic acids promote live-weight gains, but it is not possible to relate our observations, for which the technique of infusing acids into the rumen was used, with those obtained with changes in the physical condition of natural foods, since information is not available on the relative caloric efficiency of the production in the rumen of individual fatty acids from food constituents. In explaining the effect of the physical condition of the diet on the growth performance of animals, it should also be recognized that the digestibility of dietary constituents is altered and probably also the proportion of energy absorbed as fatty acids. Such factors could offset or enhance any effect due to a change in the relative proportions of the fatty acids produced in the rumen. Shaw *et al.* (1960) obtained a growth response in steers given a diet of lucerne hay, maize and linseed-oil meal, in the ratio of 1:1:0·4, whereas Blaxter & Graham (1956) observed no difference with sheep in the net energy value of dried grass when it was given finely ground or in the long form.

SUMMARY

1. Two experiments were made with four non-lactating Friesian heifers given a basal diet of hay, dairy cubes and decorticated groundnut meal. Each animal received, over successive periods of 35 days, a continuous intraruminal infusion of water or of an aqueous solution of acetic, propionic or butyric acid, according to a Latin square design. The acids supplied 3500 kcal/day in Expt 1 and 5500 kcal/day in Expt 2. Measurements were made of the effects of the infusions on nitrogen retention, change in empty-body weight, voluntary food intake, digestibility, volatile fatty acids in rumen liquor, and certain blood constituents.

2. The infusion of acetic acid depressed the pH of the rumen liquor slightly, decreased the *ad lib.* intake of hay and, in Expt 2 but not in Expt 1, decreased the digestibility of the dry matter of the diet. Similar effects were not observed with the infusion of propionic or butyric acid. All the acids gave an increase in the total volatile fatty-acid concentration of the rumen liquor, with an increase in the molar proportion of the acid being infused and decreases in the proportions of the other acids. The acids had no effect on the concentration in blood plasma of glucose, but the infusion of acetic acid increased the concentration of volatile fatty acids and the infusion of butyric acid increased the concentration of β-hydroxybutyric acid and of acetone plus acetoacetic acid.

3. All the acids gave an increase in N retention in both experiments; the mean increase in Expt 1 was 5·4 g/day and in Expt 2 it was 3·7 g/day. The effects were not significant but the greatest increase occurred with the infusion of acetic acid in Expt 1. Increases in body-weight occurred in response to the infusions of all the acids, and in Expt 2 the effect was significant ($P < 0·05$) though the difference in response between

supplements was in no instance significant ($P > 0·05$); in both experiments the rate of gain was highest with the infusion of butyric acid.

4. The results are discussed in relation to the effect of type of rumen fermentation on the efficiency of utilization of food for growth.

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Italian Translation

Melanoderma Caused by Acetic Acid

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In 1929, an article by Samuel Peck was published in the journal "Arch. fur Dermatologie et Siphilis", which stated that it was possible to obtain the classical melanoderma of the rabbit injected with pyrroles and indoles by simple and repeated subcutaneous administration of a solution containing 0.8 cc glacial acetic acid per 100 cc distilled water and simultaneous exposure of the rabbits injected with this solution to sunlight or ultra-violet rays.

This fact was of such immediate concern to me that I simply could not fail to study and repeat the tests performed by Peck, since I was the first one (1) to discover the classical melanoderma and melanuria in non-albino rabbits after injection of pyrroles and pyrrole compounds and to determine the location and presence of colorless melanogen in the skin of pigmented animals (2). It is useful to recall how, later, a large group of researchers, in addition to myself, have been able to confirm, by various physical, chemical-physical and biological methods, the brilliant hypothesis of Angeli on the pyrrolic origin (genesis) of natural melanins, an hypothesis which provides a more extensive and better explanation (according to biologists) of the known facts than all others. In fact, the pyrrolic origin of melanins is now accepted by the most illustrious scientists and is cited in all biology handbooks.

It is not necessary here to enumerate the numerous meritorious studies and to mention the contrasting nature of Angeli's concept of the pyrrolic origin of melanins and that of Bloch on phenolic origin, as well as the research done in Italy and abroad which has provided ground and substance to that difficult topic of biological chemistry designated by Samuelj as "the chemistry of deprivations"(?) ("chemie der Entsagungen"). We shall also not recall here Raper's analytical and synthetic research work on phenolic indoles (tyrosine), a fact guessed by Angeli since 1916 (3) and explained and confirmed in 1927 (4). We shall also omit here studies performed by Angeli and myself, attempting to show, using phenols, adrenaline (5) and adrenaline-like compounds (6), the possible conversion, by intraorganic oxidation, of these phenols into indoles and thus into melanogen and melanins. The volume of scientific and bibliographic data, including many Italian contributions, concerning this problem is so large that it would take a very long time to give a complete citation of such data, and a detailed collection of all the studies concerning this interesting problem would now require more than one volume. I shall refer the reader, in this case, to the bibliography at the end of this article, where the few studies cited contain all the literature data needed.

It seems strange to me that such a simple aliphatic organic compound, devoid of nitrogen (melamines are known to be cyclic, nitrogen containing compounds), as acetic acid could react intraorganically like α- or β-methylindole cited by Peck. However, as a result of my own work for 10 years and work done by others, having become convinced by now that only pyrroles and indoles are able to produce immediately by intraorganic oxidation a trichoderma, melanoderma and melanuria, I also wanted to repeat the experi-

ments of Peck and to study his deductions in the light of facts acquired so far, and especially facts obtained by Angeli's school.

Peck, in his article cited above, does not contest, after the research done by Raper, that indole and pyrrole compounds can be obtained by oxidation of tyrosine or "dopa"; he thus acknowledges a fact that I have demonstrated some time ago, namely that melanins can be formed by injection of pyrroles or indoles. And thus Peck also admits that melanin could contain an indole or pyrrole ring. However, in view of the fact that, as Angeli has demonstrated(7), pyrrole is readily polymerized (in contrast to indole) to give black products which, in their atomic ratio (and also in many other physical and chemical properties), bear an enormous similarity with natural melanins, it is difficult to sustain the hypothesis that an indole nucleus is present in these products.

On the contrary, Peck excludes the possibility that pyrrole is present in a melanogen and states "the fact that pyrroles and indoles can be obtained from phenols does not permit us to affirm that the pyrrole ring is preformed in the parent substance of melanins". Now, if we think about the rather simple means which nature utilizes in building its much more complex structures, we must consider as much more probable the hematic origin of melamins derived from pyrroles preformed in the organism or originating from some hormones, such as adrenal or thyroid hormones, etc., which are readily oxidized and which, after oxidation, would lose their benzene ring (3,4). Moreover, the urine of patients with melanosarcoma, as well as urine rendered melanotic after subcutaneous injection or ingestion of pyrrole (an ingestion which I performed on myself (8)), contains a melanogen which gives indole and pyrrole reactions and which finally gives melanin under the

action of air and oxidizing agents. It would be difficult for Peck to affirm the possibility of the presence of indole nuclei or of phenols in melanogen and in melanin obtained by intraorganic oxidation of pyrroles.

Peck and other authors are also in considerable disagreement over the breakdown products of melanins, and the fault claimed in this case is that pyrrole was not obtained with the destructive means used by these authors. In fact, such a destruction (cleavage) is particularly difficult to accomplish and yields a wide variety of products according to the process employed for this purpose. It is noted in fact (8) that the action of soda induces in organic molecules oxidation and hydrolysis processes, while heating alone leads, on the contrary, to synthesis and hydrolysis processes after a certain period of time. This is the cause of the difficulty which various experimental researchers find in agreeing about the cleavage products of melanins, since among such products pyrrole, indole, scatole, nitriles, etc. have been found. If melanin is oxidized with caution and not with soda, however, numerous pyrrole derivatives are obtained (Angeli (10)).

In regard to the presence in skin of melanogens with a pyrrole structure, we can state that whereas, according to Bloch, negative results were obtained for pyrrole with pieces of skin from non-albino rabbits cut in a freezer, Rondoni (11) (a researcher of unquestionable reputation), working with the same substance on non-albino rabbits and with extracts of melanogenic organs (pyrrole oxidase) has obtained very positive results.

In the above study, Peck also states the following 2 facts: first, "the presence of melanotic granules after injection of pyrroles is significant only if it can be shown that the formation of such granules can be observed in non-albino experimental animals; second, that the black pigment

formed is derived from the injected material and cannot be attributed to the inflammatory stimulus produced by the injected pyrrole derivatives". The answer to the first fact is that is impossible to obtain a melanoderma and trichoderma in albino rabbits, and that experiments carried out by me some time ago (12) show the absence of a prepigment (melanogen) and of specific oxidases in the same pigment. In regard to the second fact, it cannot be excluded that the organism reacts in an entirely different manner to the stimulus produced by the foreign substance, whatever it may be, but that pyrroles and indoles behave in such a way so as to produce melanouria, melanoderma and trichoderma even if the test animal is exposed to diffuse laboratory light, whereas Peck himself admits that the results obtained by injection of acetic acid are negative if the test subject is not exposed to the action of sunlight or of ultraviolet rays. This is the main difference. It is also not possible to ascribe to pyrrole a simple stimulating action, not only because such an action should then be admitted for a wide variety of substances which under the same conditions should produce the same phenomena in the skin of injected animals, but also because I have demonstrated that the injection of melanogenic substances is initially accompanied by the appearance of melanogens which can also be readily and automatically converted into melanin (under the action of air and enzymes). These melanogens exhibit a cyclic structure very similar to that of the injected substance. On the contrary, Peck does not draw any conclusions in favor of this important complement to the melanogenic action of acetic acid, with regard to the results of urine analyses. In addition, I also wish to recall that I myself (12) have obtained, by ingestion of pyrrole, a melanouria with positive pyrrole reactions, including the classical test of Thormahlen.

On the basis of all these facts, we can exclude the stimulus reaction alone; if such a stimulus is admitted for pyrrole and indole, we would have to attribute to it the melanogenic action of tyrosine, "dopa", adrenaline, etc., whereas the opposite has been demonstrated (13). If the substance injected is capable of reacting chemically with oxidases of the subcutaneous tissue to give a melano-and trichoderma without the direct action of other agents, in view of the very close and specific relationship between enzymes and the reacting substance (reagent), such a substance must be considered as a melanogen. Therefore, the melanogen would be a substance having a rather simple pyrrole structure, also because my numerous studies of pyrrole derivatives have shown that the more complex the pyrrole compound, the less positive is Thormahlen's reaction and the more difficult is the intraorganic oxidation of the compound; the more farther removed from pure pyrrole is the compound, the more alien it is to the animal organism.

In his study, Peck does not mention trichoderma, and I insist on this point. It is noteworthy, instead, that melanogenic substances, in addition to causing melanoderma, are capable of offering excellent control, as I have shown elsewhere (14), by strongly coloring the hair dark black in the injection areas (dark areas, never white), a fact which strongly supports the great affinity of pyrrole towards pigments of the Malpighian layer and its participation in the trichomelanogenic mechanism of hair.

Experimental Part

Two rabbits, with grey fur and each weighing about 2 kg, were given a subcutaneous injection of 2 cc of the acetic acid solution suggested by Peck. The urine, previously analyzed, did not disclose anything abnormal. The

rabbits were kept on a grass diet during the entire test period, since it was noted at other times that feeding a bran-based diet leads to a positive Ehrlich diazo reaction (test) in the urine.

For the sake of brevity, I shall not reproduce here my laboratory notebook, showing that the injections were repeated on 15 consecutive days, then suspended for 3 days, and again resumed for 10 more days. The rabbits were kept in a room exposed to natural diffuse light.

During this entire period, not even a trace of melanuria, melanoderma and trichoderma was noted. The urine remained constantly normal, the Ehrlich and Thormalen reactions were both negative, and the oxidation reactions were strong and probative enough to cause precipitation of melanin, as in the case of artificial melanotic urines (after injection of pyrrole).

Not being satisfied with my work, I asked Prof. L. Leinati, a histologist (whom I wish to thank here), to perform sections of the skin areas injected and cut in a freezer; nothing was found in these sections which could indicate a hyperpigmentation of the tissues, which at other times, after pyrrole injections, were tainted with melanin granules.

How can we then explain the melanoderma obtained by Peck? I believe that it can be explained as a defensive stimulus due to a reaction of corium tissues against an alien (foreign) body, and perhaps also as the action of acetic acid on the corium melanogen as a result of the modified ambient reaction of the tissue caused by the acid.

Therefore, I believe that Peck could even have done without any acetic acid for the following reason. If a small piece of the dark fur of a rabbit is removed and the animal is left exposed to light (even diffuse laboratory light is sufficient), we can observe that the exposed area, devoid of fur

(hair), gradually undergoes a self-pigmentation for defensive reasons. And if we expose ourselves to the sun on beaches or mountains, or to ultraviolet rays of a standard mercury vapor lamp, don't we see a pigmentation of exposed areas? There is even a better reason to expect such a pigmentation on the fur of a rabbit no longer covered by his fine, dense and dark hair. A rabbit injected with acetic acid does not assume a color when exposed to laboratory light, whereas a rabbit injected with pyrrole takes on a color and also exhibits a strong trichoderma. We can thus infer that the melanoderma observed by Peck must be attributed almost exclusively to the action of light. If Peck had used α- or β- methyl indole under the same conditions, he probably would not have needed such an intensity of light.

Thus, I believe that the principle of a cyclic and pyrrolic origin of natural melanins is even further strengthened. On the contrary, if Peck's work had not been disproved, it could have led us astray along the path of future research and could have generated some certainly unfavorable doubts about scientific progress in this vast and difficult problem of biological chemistry.

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ISTITUTO DI CHIMICA ORGANICA E FARMACEUTICA
DELLA R. UNIVERSITÀ DI CAMERINO

"Sulla melanodermia provocata da acido acetico,,

Prof. PIETRO SACCARDI

Biochim. e terap. sper. 18: 335-340, 1931)

Nel 1929, nell'« Arch. für Dermatologie et Siphilis » è comparso un lavoro di Samuele Peck ove si affermava la possibilità di ottenere la classica melanodermia del coniglio iniettato di pirroli ed indoli per semplice e replicata propinazione sottocute di una soluzione contenente cc. 0.8 di acido acetico glaciale per 100 di acqua stillata e contemporanea esposizione dei conigli iniettati alla luce solare od a quella dei raggi ultravioletti.

Il fatto mi riguardava troppo da vicino per tralasciare di studiare e di ripetere le esperienze di Peck, essendo stato io per il primo (1) a scoprire la classica melanodermia e melanuria dei conigli, non albini, dopo iniezioni di pirroli e composti pirrolici ed a constatare la localizzazione e la presenza di melanogeno incoloro nella pelle degli animali pigmentati (2). E' utile ricordare come, dipoi, una larga schiera di ricercatori, oltre a me, abbiano per diverse vie, fisiche, chimico-fisiche e biologiche, confermata la geniale ipotesi dell'Angeli sulla genesi pirrolica delle melanine naturali, ipotesi che più e meglio di ogni altra — a detta dei biologi — fornisce la ragione dei fatti conosciuti. La genesi pirrolica delle melanine è infatti ormai ammessa dai più illustri scienziati e citata in tutti i Trattati di Biologia.

Non è qui il caso di enumerare i molteplici lavori in merito, il contrasto fra la tesi di Angeli, della genesi pirrolica delle melanine, e quella di Bloch, della genesi fenolica, e le ricerche che in Italia ed all'estero hanno dato campo e materia a questo difficile argomento della Chimica Biologica chiamato dal Samuelj « Die Chemie der Entsagungen ». Come pure è superfluo ricordare le ricerche analitiche e sintetiche del Raper sugli indoli da fenoli (tirosina), fatto questo da Angeli intuito fino dal 1916 (3) e spiegato e confermato nel 1927 (4). Tralascio ancora i lavori di Angeli e Conici tendenti a dimostrare su fenoli, adrenalina (5) e adrenalino-simili (6) la possibilità del passaggio, per ossidazione intraorganica, dai fenoli suddetti a indoli, e quindi a melanogeno e melanine. E' tale e così copioso il materiale scientifico e bibliografico inerente a questo problema, fra cui molto Italiano, che lunghissimo sarebbe il citarlo tutto, come del pari non basterebbe ormai un volume a raccogliere dettagliatamente tutti i lavori a questo interessante problema inerenti. Rimando, nel caso, nella bibliografia al termine di questa Nota ove, nei pochi lavori citati, havvi tutta la letteratura desiderabile.

Parevami perciò strano che un composto organico grasso così semplice, privo di azoto, come l'acido acetico, (le melanine sono notoriamente sostanze azotate, a

struttura ciclica) dovesse reagire intraorganicamente come l'alfa od il beta metilindolo citato dal Peck. Ma essendomi ormai persuaso attraverso il decennale mio lavoro e quello di altri, che solo pirroli e indoli potessero, in modo immediato, per ossidazione intraorganica, fornire trico, melanodermia e melanuria, pure volli ripetere l'esperienza di Peck e studiare le sue deduzioni al lume dei fatti fin quì acquisiti specialmente dalla Scuola di Angeli.

Il Peck pertanto, nella sua Nota sopra citata, non contesta, dopo le ricerche del Raper, che da tirosina o « dopa » si possano ottenere per ossidazione composti indolici e pirrolici, per cui l'A. riconosce quello che da tempo io ho dimostrato, e che cioè iniettando pirroli o indoli si possono aver melanine. E con ciò Egli ammette anche che la melanina conterrebbe un anello indolico o pirrolico. Ma dato il fatto, come Angeli ha dimostrato, (7) che il pirrolo, a differenza dell'indolo, facilmente si polimerizza a dare dei neri che, anche nel rapporto atomico — oltre che a tante altre proprietà fisiche e chimiche — hanno enorme analogia colle melanine naturali, l'ipotesi del nucleo indolico presente nelle stesse, per questo e per quanto esporrò più sotto, è difficile a sostenersi.

Il Peck esclude invece che il pirrolo esista nel melanogeno, e dice « il fatto che da fenoli si possono avere pirroli e indoli, non autorizza ad affermare che l'anello pirrolico sia preformato nella sostanza madre delle melanine ». Ora, se si pensa di quali mezzi assai semplici Natura si serve per costruire i suoi edifici, anche più complessi, è da ritenere ancora molto probabile la genesi ematica delle melanine derivate da pirroli preformati nell'organismo o provenienti da alcuni ormoni: surrenale, tiroideo ecc., che facilmente ossidabili ed ossidati perderebbero il loro anello benzolico (3 e 4). Tanto più poi che le orine di ammalati di melanosarcomi, come quelle rese melanotiche dopo iniezioni sottocute o ingestione di pirrolo, — ingestione su me stesso (8) operata — contengono un melanogeno che presenta le reazioni degli indoli e dei pirroli, e che finisce, per azione dell'aria e degli ossidanti, per dare melanina. Sarebbe difficile al Peck l'affermare la possibilità della presenza di nuclei indolici o di fenoli nel melanogeno e nella melanina ottenuti per ossidazione intraorganica di pirroli.

Peck ed altri Autori sono inoltre assai discordi sui prodotti di scissione delle melanine, la cui colpa sarebbe quella di non fornire pirrolo con i mezzi di demolizione da loro adoperati. Infatti tale demolizione è particolarmente ardua a compiersi e fornisce i più svariati prodotti a seconda del processo adoperato. E' noto infatti (8) che l'azione della soda induce nelle molecole organiche processi di ossidazione e di idrolisi, il riscaldamento da sole provoca invece processi di sintesi e di idrolisi ad un tempo. Ecco la causa delle difficoltà che i diversi sperimentatori trovano nel mettersi d'accordo sui prodotti di scissione delle melanine, per cui si è trovato, fra altro, pirrolo, indolo, scatolo, nitrili, ecc. Ma se la melanina si ossida con cautela e non con soda, si ottengono abbondanti derivati pirrolici (Angeli) (10).

Sulla presenza poi di melanogeni a struttura pirrolica nella pelle, posso affermare che se pezzi di pelle di coniglio non albino, tagliati al congelatore han dato, secondo il Bloch, risultato negativo, col pirrolo, uno sperimentatore di indubbia fama, il Rondoni (11) operando colla stessa sostanza, e su conigli non albini con estratti di organi melanogeni, (pirrolossidasi) ha ottenuti risultati ben positivi.

Nello stesso lavoro succitato poi il Peck afferma due fatti: Primo che « l'aver granuli melanotici dopo iniezioni di pirroli ha valore solo se si può dimostrare che tale formazione si osserva in animali sperimentati non albini; secondo che il pigmento nero formato derivi da materiale iniettato e non debba attribuirsi allo stimolo

infiammatorio dei derivati pirrolici iniettati ». Al primo fatto risponde esaurientemente l'impossibilità di ottenere melano e tricodermia in conigli albini, che da esperimenti da tempo da me fatti (12) mostrano di mancare e del propigmento (melanogeno) e delle ossidasi specifiche del pigmento stesso. Pel secondo fatto non è da escludersi che l'organismo reagisca allo stimolo della sostanza estranea, qualunque essa possa essere, tutt'altro, ma i pirroli e gli indoli si comportano dando anche melanuria, melanodermia e tricodermia anche se l'animale in esperimento è tenuto alla luce diffusa del Laboratorio, mentre il Peck stesso ammette che i risultati ottenuti per iniezioni di acido acetico sono negativi, se non si espone il soggetto alla luce solare od a quella dei raggi ultravioletti. Ecco la differenza capitale. Per il pirrolo poi non è possibile invocare una pura azione di stimolo, non solo perchè allora essa si dovrebbe ammettere anche per le più svariate sostanze, che nelle stesse condizioni dovrebbero dare i medesimi fenomeni a carico della pelle degli animali iniettati, ma anche perchè ho dimostrato che iniettando sostanze melanogenetiche compaiono nelle urine dei melanogeni anch'essi facilmente ed anche automaticamente (azione dell'aria e dei fermenti) trasformabili in melanina. Questi melanogeni mostrano costituzione ciclica molto affine a quella della sostanza iniettata. Il Peck invece nulla conclude a vantaggio di questo importante complemento all'azione melanogenica dell'acido acetico, relativamente al risultato delle analisi delle urine. Inoltre ricordo ancora che io stesso (12) per ingestione di pirrolo, ebbi melanuria con reazioni pirroliche positive quali la classica prova del Thormählen. Tutto questo esclude la sola reazione di stimolo; il quale, se si ammette per pirrolo ed indolo, ad esso dovrebbe attribuirsi l'azione melanogenetica della tirosina, « dopa », adrenalina, ecc., mentre è dimostrato il contrario (13). Se la sostanza iniettata è capace di reagire colle ossidasi del tessuto sottocutaneo, chimicamente, a dare melano e tricodermia senza l'azione diretta di altri agenti, data la relazione strettissima e particolare fra enzime e sostanza reagente, essa deve essere considerata un melanogeno. Il melanogeno sarebbe adunque una sostanza avente struttura pirrolica assai semplice, anche perchè i molteplici studi miei su derivati pirrolici hanno dimostrato che più complesso è il composto pirrolico e meno positiva è la reazione di Tormählen, e tanto più difficile è l'ossidazione intraorganica del composto. E tanto più ci si allontana dal pirrolo puro e tanto più il composto si mostra estraneo all'organismo animale.

Nel suo lavoro il Peck non parla di tricodermia: io insisto su questo punto. E' notevole invece che le sostanze melanogenetiche oltre che melanodermia hanno la possibilità di presentare l'ottimo controllo, come altrove ho dimostrato (14), di tingere fortemente il pelo delle aree iniettate (aree scure, mai albine) in nero intenso, fatto che molto suffraga la familiarità grande del pirrolo verso i fermenti dello strato malpighiano e la partecipazione di questo al meccanismo tricomelanogenetico del pelo.

PARTE SPERIMENTALE

A due conigli del peso di circa Kg. due caduno, dal mantello grigio, presi ad iniettare sottocute cc. 2 della soluzione acetica suggerita dal Peck. Le orine previamente analizzate, non mostrarono nulla di anormale. I conigli furono mantenuti, durante tutti gli esperimenti a dieta di erba, avendo notato altre volte, che l'alimentazione a base di crusca fa comparire nelle orine positiva la diazoreazione di Ehrlich.

Per brevità non riproduco il quaderno di Laboratorio dal quale risulta che le iniezioni furono ripetute per 15 giorni consecutivi, sospese per 3 e riprese dipoi per altri 10 giorni. I conigli furono mantenuti in una stanza alla luce diffusa naturale.

Durante tutto questo periodo i noti fenomeni di melanuria, melanodermia e tricodermia, neppure comparvero in tracce. Le orine si mantennero costantemente normali, negativa la diazoreazione di Ehrlich, quella di Thormälen, e le rezioni di ossidazione così forti e probative come nel caso di orine melanotiche artificiali (dopo iniezioni di pirrolo), tanto da precipitare della melanina.

Non contento però dell'opera mia feci eseguire all'istologo, Prof. L. Leinati, che mi è grato ringraziare, delle sezioni della pelle iniettata tagliata al congelatore, ma nulla trovò che potesse accennare ad una iperpigmentazione di quei tessuti che altre volte, dopo iniezioni di pirroli, erano infestati di granuli di melanina.

Come si spiega allora la melanodermia ottenuta dal Peck?

Credo si possa spiegare collo stimolo difensivo dovuto ad una reazione dei tessuti del derma contro il corpo estraneo e forse anche ad un'azione dell'acido acetico sul melanogeno del derma in conseguenza della mutata reazione ambientale del tessuto operata dall'acido.

Però io penso che dell'acido acetico il Peck avesse potuto anche farne a meno: Se si spela un poco del mantello scuro di un coniglio e si lascia l'animale alla luce — basta anche quella diffusa di Laboratorio — si osserva che l'area scoperta, piano-piano, per ragione difensiva, mancando il pelo, si va pigmentando da sè. E se ci esponiamo al sole delle spiagge o dei monti od ai raggi ultravioletti di una comune lampada a vapori di mercurio, non vediamo pigmentarci nelle aree scoperte? A maggior ragione avverrà del mantello del coniglio non più coperto dal fino e fitto suo pelo scuro. Il coniglio, iniettato di acido acetico, non si colora alla luce di Laboratorio, quello iniettato di pirroli si colora, e mostra anche forte tricodermia. Si può quindi ben inferire che la melanodermia osservata dal Peck si debba attribuire quasi esclusivamente all'azione della luce. Veda il Peck che operando nelle stesse condizioni con alfa o beta metilindolo, probabilmente non avrà bisogno di tale intensità di luce.

Credo così che resti ancor più e meglio fissata la tesi della genesi ciclica e pirrolica delle melanine naturali. Chè se invece il lavoro del Peck fosse rimasto senza smentita avrebbe potuto fuorviare la traccia delle future ricerche ed ingenerare dei dubbi non certo favorevoli al progresso scientifico di questo vasto e difficile argomento della Chimica Biologica.

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BIOSYNTHESIS OF FATTY ACIDS AND CHOLESTEROL IN A FRESH-WATER CARP, «SCARDINIUS ERYTHROPHTHALMUS» FROM SODIUM ACETATE-1-^{14}C

BY

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(3 figures)

INTRODUCTION

While studying the fatty acid composition of *Scardinius erythrophthalmus* L. in May and October samples, the authors (SAXENA and ZANDEE, 1968) tried to isolate squalene and cholesterol from the unsaponifiable lipid fraction. However the squalene fraction was found negative by thin layer chromatography and hence it was thought that this Fish synthesizes cholesterol on such a small scale that squalene is indetectable. Moreover certain unsaturated branched chain fatty acids were also found in the fatty acids isolated from this Fish.

It has become an universally accepted fact that fatty acids and cholesterol in animals are synthesized from lower units such as acetate; the latter *via* mevalonate and cyclization of squalene. Hence the present study was undertaken to ascertain the biosynthesis of fatty acids and sterols with the help of sodium acetate-1-^{14}C.

A comprehensive account of litterature dealing with various aspects of fatty acids in Fishes has recently been given by ACKMAN (1967) and ACKMAN and HOOPER (1968). The chemical structure of fatty acids from fish oils has also been studied (KLENK and BROCKERHOFF, 1957, 1958; KLENK and EBERHAGEN, 1957; STOFFEL *et al.*, 1958; STOFFEL and AHRENS, 1958, 1960; ACKMAN and CASTELL,

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1966; ACKMAN, 1967; ACKMAN *et al.*, 1967; ACKMAN and KE, 1968 and ADDISON *et al.*, 1968). All the studies so far made lead to the conclusion that Fishes possess more unsaturated fatty acids of higher chain lengths and that they belong to oleic (ω9), linoleic (ω6) or linolenic (ω3) acids in structure at their methyl ends. KAYAMA and TSUCHIYA (1962) indexed unsaturated fatty acids of Fishes studied till that time and elucidated biosynthetic pathways for polyunsaturated fatty acids. The concensus of opinion is that fisch polyunsaturated fatty acids mostly belong to the category of linolenic acid (ω3).

Our present knowledge on the mechanism of fatty acid biosynthesis is mainly based upon mammalian tissues (CORNFORTH, 1959; WAKIL, 1961; MEAD, 1961; MOHRHAUER and HOLMAN, 1965; CHRIST, 1968; MARCEL *et al.*, 1968 and QUAGLIARIELLO *et al.*, 1968). Biosynthesis of fish polyunsaturated fatty acids on a very limited scale has been studied by MEAD *et al.* (1960) and KAYAMA *et al.* (1963).

The study of sterol synthesis in Fishes has been rather scarce in comparison to that of Mammals (for review see POPJÁK and CORNFORTH 1960; BLOCH, 1965 and KRITCHEVSKY, 1967). Among Invertebrates it is known that some are incapable of synthesizing sterols and consequently utilize the dietary sterols. This is true for most of the Arthropods (ZANDEE, 1966, 1967). Among Molluscs some of the species are capable while others are not able to synthesize sterols from acetate (VOOGT, 1967*a* and *b*, 1968*a* and *b*).

In dog fish and rainbow trout SCHWENK *et al.* (1955) found a very low rate of cholesterol synthesis after the injection of sodium acetate-1-^{14}C. Further, they reported that besides cholesterol digitonin also precipitated some other highly active materials which they termed "Higher Counting Companions or HCC„. Later SCHNEIDER *et al.* (1957) thought that a part of HCC were comparable to lanosterol, an intermediate between squalene and cholesterol. BLONDIN *et al.* (1966) used mevalonic acid-2-^{14}C both in *vivo* and *in vitro* experiments with fresh and salt water bass and detected the synthesis of both squalene and sterols; however under similar conditions of experiment the fish liver homogenates showed a slower rate of incorporation of ^{14}C in comparison to that of rat liver. They also concluded that pathway of sterol synthesis *in* Fish and Mammals appeared to be similar. KAYAMA and TSUCHIYA (1965) in leopard shark reported the incorporation of acetate-1-^{14}C in pristane and squalene.

MATERIAL AND METHODS

A young fresh water carp, *Scardinius erythrophthalmus L.*, (family: Cyprinidae, sub-family: Abraminae) weighing 57 g was caught in December two days before the investigation and was acclimatized to laboratory conditions at 14° C without feeding. The fish received the total dose of 58.28μC (0.24 mg) of sodium acetate-1-^{14}C by intraperitoneal injection and it was kept alive for 20 hours in a small dessicator through which a slow stream of CO_2 free air was passed and collected at the other end for determining the activity in expired carbon dioxide. The expired carbon dioxide was trapped in 150 ml of ethanol amine scintillation fluid (50 ml ethanol amine with 5 g PPO(1), 400 ml ethyleneglycol monoethylether and 500 ml of toluene) and the activity was measured from the fourth to the twelfth hour at every two hour interval. Each time the scintillation fluid was renewed.

After 20 hours of incubation *in vivo* the fish was killed by cutting into pieces and was homogenized in chloroform methanol (2 : 1). The homogenate was filtered and the filtrate was washed with water, evaporated and dried over phosphorus pentaoxide under vacuum. This yielded 3 504.9 mg of total lipid mixture (6.15 % of body weight). The radioactivity in the total lipid mixture was measured in a scintillation fluid of 15 ml toluene with PPO and POPOP(2) (2.5 liters of toluene with 10 g PPO and 125 mg POPOP). The separation of lipid classes was done by silicic acid thin layer chromatography (SKIPSKI *et al.*, 1965). The chromatograph plates were scanned for radioactivity by automatic, two-dimentional chromatography scanner (Berthold). The distribution of activity in the lipid classes was determined by suspending each lipid spot in Cabosil (4 % by weight added to PPO-POPOP scintillation fluid).

The total lipid mixture was saponified in sufficient quantity of 1.5 N KOH in 80 % methanol at a reflux temperature for 4 hours under an atmosphere of nitrogen. Unsaponifiable fraction from the hydrolysate was extracted with ether after dilution with water. The unsaponifiable lipids after drying amounted to 335.3 mg (0.59 % of body weight). The remaining fraction was acidified

(1) 2,5 diphenyloxazole.
(2) 1,4-bis-2-[(5-phenyloxazolyl)]-benzene.

with HCl, decolourised with activated charcoal and extracted with petroleum ether which yielded 1 356.12 mg (2.37 % of body weight) fatty acids (saponifiable lipids). Methyl esters of fatty acids were analysed on a gas chromatograph machine (Julian Becker, Delft) equipped with catharometer detector and 2 meter long columns filled with chromosorb 60-80 mesh, coated with 20 % PEGA and 2 % phosphoric acid. The temperature of the columns was 190° C and the helium as carrier gas was used at a flow rate of 50 ml per minute. On account of overlapping of certain peaks, the fatty acid mixture was separated into saturated and unsaturated fractions (GOLDFINE and BLOCH 1961). Each fatty acid methylester eluted from the column was simultaneously examined for radioactivity. The activity was measured by passing the fatty acid methylesters through a Packard Tri-Carb combustion furnace (Model 325; cupric oxide heated to 750° C and attached to a water trap) for combusting into carbon dioxide which was lead through the antracene filled flow cell placed in the Packard Tri-carb Liquid Scintillation Spectrometer (Model 2002). The scintillation apparatus was attached to the recorder through a Packard ratemeter model 280 A. The effluent carbon dioxide was trapped in ethanol amine scintillation fluid and was also examined for activity for determining the efficiency of the system. The retention time of carbon dioxide in the antracene flow cell was 0.14 minute. The area of each active peak corresponding to the respective fatty acid ester peak was converted into counts per minute (cpm). Since the scintillation apparatus had an efficiency of 60 % the distribution of activity in each active acid was converted to disintegrations per minute (dpm/μl) of total fatty acid mixture.

The unsaponifiable lipids were subjected to aluminium oxide column chromatography (SCHNEIDER *et al.*, 1957) and all the eluates from the column were tested for squalene, wax alcohols, hydrocarbons and sterols by thin layer chromatography (TLC). The supposed squalene fraction after evaporation yielded 29.8 mg (0.05 % of body weight) and its activity was measured. However, when tested by TLC the squalene spot could not be detected though other hydrocarbons were present. Subsequently it was purified as thiourea adduct (CHAYKIN, 1966) which also did not precipitate squalene.

The sterol fraction was evaporated and gave dry weight of 72.7 mg (0.13 % body weight). After determining the activity of total

sterols, 3 β-sterols (cholesterol) were isolated as digitonides (WINDAUS, 1909) while the remaining sterols contained in the filtrate were dried and their activity was counted. The digitonides on splitting with pyridine and precipitating digitonin with excess of ether yielded cholesterol which was filtered and eventually evaporated. The dry residue of cholesterol weighed 42.1 mg (0.07 % of body weight) and was checked for radioactivity. The cholesterol was purified eight times in methanol untill the activity count became constant.

The purity of cholesterol was checked by gas chromatography (Julian Pecker, Model 2300, Delft, The Netherlands) on selective and non-selective columns. The apparatus was equipped with flame ionization detectors and two glass columns of 1.21 meter length (inner diameter 4 mm). Each column was filled with gas chrom Q (60-100 mesh). The stationary phase in one of the columns was 0.8 % neopentyl glycol succinate (NPGS) as the selective phase and in the other 0.6 % SE 30 (silicon polymer) as the non-selective phase. The carrier gas was nitrogen while the temperature of columns and injection point were 230° C and 260° C respectively.

RESULTS

The results of radioactivity in the expired carbon dioxide (Fig. 1) reveal the maximum expiration of ^{14}C about 8 hours after the injection of sodium acetate-1-^{14}C. The amounts of various lipid fractions obtained after extraction, and their activity are shown in Tables I and II respectively. The distribution of activity in various lipid classes (Table III) after separation on a thin layer plate was maximum in phospholipids (1 570.8 dpm/mg lipid mixture) while free cholesterol had only 4.7 dpm/mg total lipid mixture. Except for triglycerides, other lipid classes also had low activities.

The activity in the total lipid mixture was 2 237.9 dpm/mg (Table II). This means an incorporation of 7 843 615.7 dpm (6.1%) in the total lipid mixture from the dose (58.28 μC) which was administered to the fish. Since the activities in fatty acids (saponifiable lipids) and the unsaponifiable lipids were 2 422.3 and 955.5 dpm/mg respectively, the corresponding total activities in them should be 3 284 929.5 (1.48 μC) and 320 379.2 dpm (0.14 μC). Thus the fatty acids and the unsaponifiable lipids incorporate 41.6 % and 4.0 %

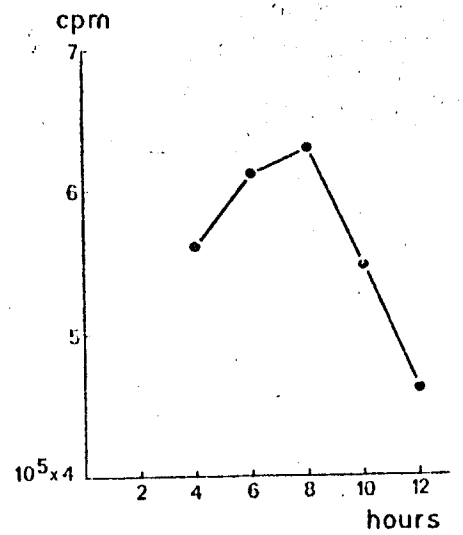

FIG. 1. *Incorporation of ^{14}C in the expired carbon-dioxide of* Scardinius erythrophthalmus.

TABLE I. *Quantities of lipids isolated from* Scardinius erythrophthalmus *after the injection of sodium acetate-1-^{14}C.*

Lipid fraction	Weight in mg	% body weight
Fish	57 000.0	—
Total lipid mixture	3 504.9	6.15
Unsaponifiable lipids	335.3	0.59
Fatty acids (saponifiable lipids)	1 356.12	2.37
Total sterols	72.7	0.13
"Squalene-hydrocarbon fraction"	29.8	0.05
3 β-sterols (Cholesterol)	42.1	0.07

respectively of the activity of total lipid mixture. Similarly the activity in "Squalene-hydrocarbon„ fraction was 3 491.0 dpm/mg which corresponds to a total activity of 104 031.8 dpm or 32.7 % of the activity of unsaponifiable lipid mixture. In contrast the total sterols incorporate only 4.0 % (12 977.0 dpm) activity of the unsaponifiable lipids. The cholesterol before and after recrystallization possessed an activity of 145.9 dpm/mg and 118.1 dpm/mg respectively. Hence the total activity after recrystallization amounts to 4 972.0 dpm which is 1.5 % of the activity of unsaponifiable

TABLE II. *Radioactivity in the lipid fractions isolated from* Scardinius erythrophthalmus *after the injection of sodium acetate-1-*^{14}C.

Lipid fraction	dpm/mg	Total activity in dpm	% of total dose (58.28 μC)
Total lipid mixture	2 237.9	7 843 615.7	6.06
Fatty acids	2 422.3	3 281 929.5	2.54
Unsaponifiable lipids	955.5	320 379.2	0.24
Total sterols	178.5	12 977.0	0.01
Cholesterol (crude)	115.9	6 142.4	0.005
Cholesterol (Recrystallization)	118.1	4 972.0	0.007
Remaining sterols	212.3	—	—
"Squalene hydrocarbon"	3 491.0	104 031.8	0.08
Hydrocarbons and waxes	5 712.7	—	—

TABLE III. *Distribution of activity in the lipid classes after the injection of sodium acetate-1-*^{14}C *in* Scardinius erythrophthalmus.

Lipid class	dpm/mg of lipid mixture
Phospholipids	1 570.8
Monoglycerides	35.6
Cholesterol	4.7
Diglycerides	20.5
Free fatty acids	53.1
Triglycerides	396.6
Cholesterolesters	60.4

lipid fraction. This also shows that the loss in activity after recrystallization was only 27.8 dpm/mg or a total of 1 170.4 dpm.

The study of the gaschromatograms of the cholesterol fraction (Fig. 2) on both selective and non-selective phases on comparison with the reference sample showed that it mainly consisted of cholesterol. However, it was accompanied with a trace amount of cholestane.

The percentage composition of individual fatty acids and their activity as revealed by the gas liquid chromatography and anthracene flow cell scintillation spectrometer respectively are shown in Table IV. The totals of *iso* branched chain, *anteiso*-branched chain and unsaturated fatty acids (unsaturated *anteiso* inclusive), are 1.84, 1.78 and 72.42 percents respectively. The unsaturated C_{22} *anteiso* branched fatty acids are present as 6.96 percent. Percentages of principal fatty acids and their comparison with previous

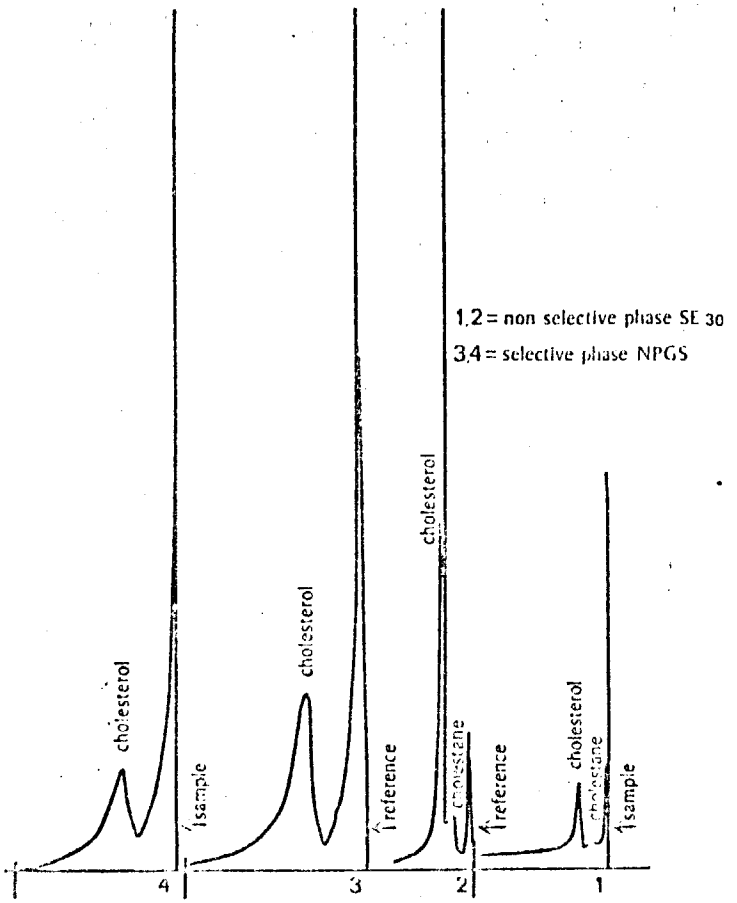

FIG. 2. *Gas chromatogram of the cholesterol fraction of* Scardinius erythrophthalmus *on selective and non-selective phases.*

results are given in Table V. The analysis of fatty acid mixture after hydrogenation revealing besides the normal unsaturated fatty acids also showed the presence of unsaturated C_{22} *anteiso* branched chain fatty acids (Fig. 3). The activity of individual fatty acids is shown in dpm/μl of total fatty acid mixture which on summing up amounts to 2 179.34 dpm/μl.

TABLE IV. *Percentage composition and dpm's of individual fatty acids in a fresh water fish,* Scardinius erythrophthalmus *from three unhydrogenated and two hydrogenated gas-liquid chromatograms.*

Shorthand designation	Percentage	dpm/µl of total fatty acid mixture	Shorthand designation	Percentage	dpm/µl of total fatty acid mixture
12 : 0	0.09	—	17 : 2	0.47	18.92
13 : 0	0.04	2.62	18 : 0 *iso*	0.36	12.38
14 : 0 *iso*	0.07	6.18	18 : 0	3.30	181.54
14 : 0	1.42	25.70	18 : 1	24.36	529.50
14 : 1	trace	67.09	18 : 2	11.36	102.51
14 : 2	trace	2.00	18 : 3	0.63	—
14 : 4	0.05	—	18 : 4	5.35	—
15 : 0 *iso*	0.38	—	19 : 0 *neo*	2.39	—
15 : 0 *anteiso*	0.27	—	19 : 0	0.09	—
15 : 0	0.49	23.62	19 : 2	0.62	—
16 : 0 *iso*	0.23	14.58	20 : 0	0.81	17.52
16 : 0	13.83	738.84	20 : 1	1.36	30.17
16 : 1	10.32	118.26	20 : 2	0.88	5.80
16 : 2	0.20	136.26	20 : 3	1.71	—
16 : 3)	1.26	—	20 : 4	5.81	—
17 : 1)			22 : 0 *anteiso*	0.82	5.65
16 : 4	1.62	23.83	22 : 1 *anteiso*	2.36	41.49
17 : 0 *neo*	0.91	—	22 : 3 *anteiso*	1.71	—
17 : 0 *iso*	0.80	—	22 : 4 *anteiso*	2.89	78.85
17 : 0 *anteiso*	0.69	—			
17 : 0	0.58	—			

TABLE V. *Percentages of the principal fatty acids in the Fish,* Scardinius erythrophthalmus *in December sample* (compared with previous results : SAXENA and ZANDEE, 1968).

	Saturated acids[a]				Unsaturated acids			
Number of C-atoms	C_{14}	C_{16}	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20}
May	1.7	12.2	2.0	—	—	15.4	45.0	7.5
October	5.1	29.5	9.6	0.7	—	18.2	18.5	0.9
December	1.5	14.1	3.7	0.8	trace	13.4	41.7	9.2

[a] Inclusive branched chain fatty acids.

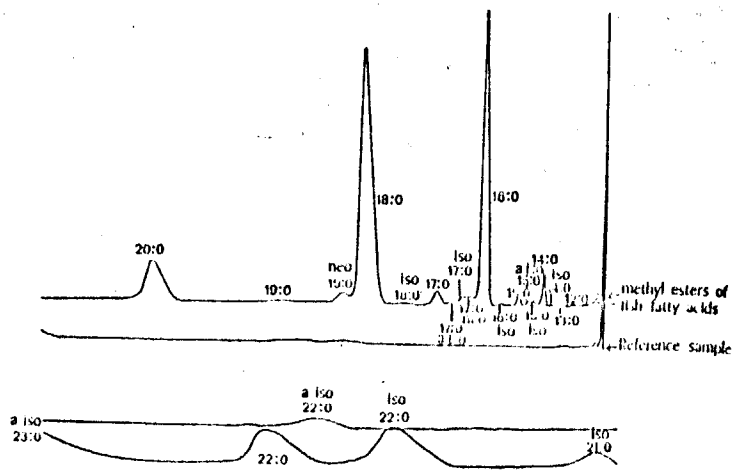

FIG. 3. *Gas liquid chromatogram of the hydrogenated sample of fatty acid-methyl ester* showing elution of unsaturated 22 : 1, *anteiso*, 22 : 3, *anteiso* and 22 : 4 *anteiso* at the position of 22 : 0 *anteiso*.

DISCUSSION

An appreciable increase in the weight of lipids is noticeable in comparison to the data of previous results (SAXENA and ZANDEE, 1968) of the same Fish (Table I). The percentages of principal fatty acids (Table V) are more near to that of May sample; moreover, with the increase in the total weight of saturated fatty acids a decrease in the total amount of unsaturated fatty acids is noticeable. Hence there appears to be an inverse relationship between the percentages of saturated and unsaturated fatty acids in this Fish. The absence of polyunsaturated fatty acids of higher chain lengths other than C_{22} may be due to perhaps starvation since in rats total inhibition of desaturation of fatty acids has been reported (ELOVSON, 1965).

The percentage composition of fatty acids in *Scardinius* shows palmitic, palmitoleic, stearic and oleic acids as components of major metabolic importance. The activity in various fatty acids is an indication of their biosynthesis; though it also depends on the quantities of the pre-existing fatty acids in depot fats. Among the branched chain fatty acids, 15 : 0 *anteiso*, 15 : 0 *iso*, 17 : 0 *anteiso*, 17 : 0 *iso*, 17 : 0 *neo* and 22 : 3 *anteiso* are inactive. Thus only the

even numbered branched chain fatty acids are active. VAGELOS *et al.* (1961) from the *in vitro* experiment with mammalian adipose tissue isolated the enzyme system, which besides the normal fatty acids, can also synthesize the odd numbered *iso* or *anteiso* acids provided a short chain odd numbered *iso* or *anteiso* fatty acyl-CoA derivative in addition to malonyl-CoA and NADPH is present.

Fishes are normally known to possess polyunsaturated fatty acids which are derived from linolenic acid (18 : 3,ω3). The present study reflects inactive linolenic acid corresponding with the inactive eicosa-tetraenoic acid (20 : 4), which (arachidonic acid)under normal conditions is synthesized by chain elongation of linoleic acid (18 : 2, ω6). High activity in the trace amount of tetradeca-mono-enoic (14 : 1) may perhaps be indicative of starting material in the biosynthesis of palmitoleic and linoleic acids. Recently ACKMAN (1967) and ADDISON *et al.* (1968) have described the fish polyunsaturated fatty acids which have the same structure at the methyl end as in the linoleic acid (18 : 2, ω6). *Scardinius erythrophthalmus* seems to be completely dependent on food for octadeca-tetra-enoic (18 : 4) and eicosa-tetra-enoic acid (20 : 4) since in the case of biosynthesis by chain elongation from inactive linoleic or linolenic acids there should have been some activity after the injection of sodium acetate-1-^{14}C. MEAD *et al.* (1960), after injecting *Tilapia mossambica* with sodium acetate-1-^{14}C found activity in arachidonic acid. Further, their degradative study of arachidonic acid showed it to be derived from inactive linoleic acid, whereas the active linoleic acid had uniform activity in its molecule indicating the total incorporation of oleic acid.

In Mammals linoleic acid (18 : 2, ω6) is the essential fatty acid and forms the parent group for polyunsaturated fatty acids though under certain exigencies of life linoleic acid, oleic acid and palmitoleic acid may be converted to polyunsaturated fatty acids (MEAD *et al.*, 1960; MEAD, 1961). Moreover in various animals it is reported that the rate of turnover of 1-^{14}C-acetate differs amongst the organs of the same animal. Fasting rat liver (MEAD and HOWTON, 1960) shows a markedly reduced incorporation of acetate-1-^{14}C.

The gas chromatogram of cholesterol fraction shows that like other Vertebrates the Fish *Scardinius* also possesses cholesterol as the chief constituent of 3 β-sterols. The presence of cholestane can not be taken as impurity since it is not found in the animal

tissue but is a saturated degradation product (FIESER and FIESER, 1959).

All the lipid fractions extracted from the Fish showed incorporation of ^{14}C. Hence the picture which emerges out the study of radioactivity is that cholesterol is synthesized in this Fish from acetate, but seems to be esterified immediately (Table III). Besides it also shows a slow rate of cholesterol synthesis as has been observed in other Fishes (SCHWENK *et al.*, 1955; BLONDIN *et al.*, 1966). The slow rate of synthesis may be attributed to the feeding habit of this Fish since besides eating phyto- and zoo-plankton, it is reported to be a carnivore as well (RUTING, 1963). Consequently the dietary sterols may be sufficing the major demand of the body for sterols.

The absence of squalene, as shown by TLC as well as thiourea adduct preparation might be the result of very slow rate of cholesterol biosynthesis. In two Gastropods, *Arion rufus* and *Buccinum undatum*, VOOGT (1967*a*,*b*) also could not detect squalene though he demonstrated that the former was capable of synthesizing 3 β-sterols.

In Mammals (SCHNEIDER *et al.*, 1957) squalene and lanosterol shortly after the injection of sodium acetate-1-^{14}C contribute activity to a major extent in the unsaponifiable lipids but after long periods the accumulation of activity is found in cholesterol. In the Fish *Scardinius* the so called squalene fraction even after 20 hours of incubation possesses 32.7 % activity of the unsaponifiable lipids whereas cholesterol shows 1.5 % activity. In most cases the crude squalene fractions are greatly polluted with hydrocarbons (see ZANDEE, 1967). The radioactivity found in this fraction of *Scardinius* may be due to the hydrocarbons only.

Our conclusion is that squalene is an obligatory precursor and we may argue that in this Fish squalene is produced in ultramicro quantities which have high turnover rates and hence can not be detected.

SUMMARY

Distribution of activity in *Scardinius erythrophthalmus* was maximum in phospholipid whereas free cholesterol was least active after the injection of sodium acetate-1-^{14}C. Percentage composition and biosynthesis of fatty acids have been studied. Certain unsaturated branched chain C_{22} *anteiso* acids are synthesized by this Fish.

A very slow rate of cholesterol synthesis has been shown to exist. The presence of squalene could not be detected.

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Humoral Conditioning for Necrosis

Part II

The Challengers

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Zusammenfassung. Eine große Anzahl, ihrer Struktur und pharmakologischen Wirkungsweise nach grundverschiedener, chemischer Substanzen können nach geeigneter Sensibilisierung mit Mastzellentladern oder Serotonin als Provokatoren für das Phänomen der akuten, konditionierten Nekrose (ACN) wirken. Unter den diesbezüglichen geprüften Verbindungen erwiesen sich neben hypertonischen Lösungen auch Essigsäure, Äthanol, Galle, Papain und Trypsin als die wirksamsten Provokatoren. Anhaltend wirkende Entzündungserreger (z. B. Krotonöl, Carrageen, Agar und viele Metallsalze) sind als Provokatoren wenig oder überhaupt nicht wirksam. Das wichtigste Charakteristicum der hochaktiven Provokatoren scheint ihre sehr starke, aber vorübergehende, gewebsschadigende Wirkung zu sein.

Die vermutliche Rolle der ACN in der Pathogenese verschiedener krankhafter Veränderungen wird kurz besprochen.

Summary. A large number of compounds, essentially different from each other in their chemical structure and pharmacologic activity, can act as challengers for the acute conditioned necrosis (ACN) phenomenon following appropriate sensitization with mast-cell dischargers or 5-HT.

Apart from hypertonic solutions, the most active challengers among those tested were acetic acid, ethanol, bile, papain, and trypsin. Long acting inflammatory irritants such as croton oil, carrageenin, agar, and many metallic salts exhibit little, if any, challenging potency. The most salient characteristic of potent challengers appears to be that they exert a very intense but evanescent damaging effect upon tissues.

The possible role of the ACN in the pathogenesis of various morbid lesions is briefly discussed.

In Part I of this study (SELYE *et al.*, 1966) we showed that after sensitization by systemic treatment with certain conditioning factors (e.g., mast-cell dischargers, histamine, serotonin), extensive skin necrosis is produced by the subcutaneous administration of normally well-tolerated doses of certain challengers (e.g., hypertonic solutions of glucose, NaCl or urea). This phenomenon of "acute conditioned necrosis" (ACN) occurs a few minutes or at the most a few hours after challenge; thereby it differs from the "delayed conditioned necrosis" (DCN) which develops much more slowly—for example, after conditioning with glucocorticoids—at sites of treatment with long-acting inflammatory irritants (SELYE, 1953, 1954).

The preceding communication was concerned with the identification of conditioning agents which sensitize for the ACN type of response and the potency of these was tested only by challenge with hypertonic solutions of urea, glucose, and NaCl. However, it soon became evident that the necrotizing action of many other tissue irritants can also be greatly enhanced by this type of conditioning. For a better understanding of the underlying pathogenic mechanisms, it was therefore deemed essential to examine numerous additional tissue irritants, essentially different in their chemical structure and pharmacologic effects, for their possible ACN-challenging potency.

Materials and Methods

950 female Sprague-Dawley rats with a mean body weight of 100 g (range 90 to 110 g) were subdivided into 95 equal groups and treated as outlined in Table 1. The following agents were used either as conditioners or as challengers:

Acetic acid. C. P. Reagent (C.I.L., Montreal, Canada).

Agar III. U.S.P., Lot No. 365 (Brickman & Co., Montreal, Canada).

Calcium chloride. $CaCl_2$ (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.).

Carrageenin. Ordinary impure commercial carrageenan. (Kindly supplied by Dr. S. D. Upham of Marine Colloids Inc., New York, U.S.A.)

Croton oil. (Brickman).

Cupric chloride. $CuCl_2 \cdot 2H_2O$ (Fisher).

Dextrin. British gum or starch gum, a polysaccharide $[(C_6H_{10}O_5)n \times H_2O]$ prepared by incomplete hydrolysis of starch (Difco Labs, Detroit, U.S.A.).

Ethanol. Ethyl alcohol (University of Montreal, Montreal, Canada).

Formalin. 40% solution of formaldehyde (Fisher).

Glucose. (Fisher).

Histamine. Histamine phosphate (Abbott Laboratories, North Chicago, U.S.A.).

5-HT. 5-hydroxytryptamine, Serotonin creatinine sulfate (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.).

Indium chloride. $InCl_3$ (Brickman).

Lead acetate. $Pb(C_2H_3O_2)\ 2 \cdot 3H_2O$ (Fisher).

Lycopodium. (Fisher).

Manganese chloride. $MnCl_2 \cdot 4H_2O$ (Fisher).

Mercury chloride. $HgCl_2$ (Fisher).

Ox bile N.F. (Fisher).

Papain. (Pharmacie Montréal, Montreal, Canada).

Polymyxin. Polymyxin-B sulfate (Abbott Laboratories).

Potassium permanganate. $KMnO_4$ (Fisher).

Sodium metaphosphate. $(NaPO_3)_6$ (Brickman).

Tannic acid. (Fisher).

Trypsin. (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.).

Tween 80. (Brickman).

Zinc chloride. $ZnCl_2$ (Fisher).

The experimental procedure was essentially the same as in the preceding paper (SELYE *et al.*, 1966) and hence need not be discussed here in detail. Suffice it to mention that the conditioning agents were injected either intravenously in 1 ml or subcutaneously in 0.2 ml of distilled water, while the challengers were administered into the deep connective tissue on the middle of the back in 2 ml of distilled water (except for croton oil which, being insoluble in water, was diluted in 2 ml of peanut oil).

Results

Since very many substances can act as conditioners or challengers for the ACN, the number of possible combinations is enormous. However, in this study, we could limit ourselves to a few standard conditioners (5-HT, polymyxin, and dextrin) since our primary object was to establish what kind of compound might possess challenging activity.

In the first experimental series (Table 1), we used a few representatives of various types of tissue irritants (mast-cell dischargers, metallic salts, polysaccharides, bile, proteolytic enzymes) more or less at random to establish approximately to what classes of agents tissues can be sensitized through the ACN-mechanism. For example, we wanted to know whether mast-cell dischargers, 5-HT and histamine, which are particularly potent conditioners, can also act as challengers. This is evidently not the case (Groups 1—9). It may be concluded therefore that the extraordinarily intense challenging potency of hypertonic solutions is not merely the consequence of their well-known mast-cell degranulating effect.

The other potential challengers were all tested only in combination with 5-HT, one of the most potent conditioners known up to now. A given dose of 5-HT is most active when introduced directly into the circulation, but comparatively small amounts are tolerated by this route; hence, in general, the subcutaneous injections were preferred. In preliminary experiments (not reported here) we first determined the highest dose of the challengers that is tolerated without producing local cutaneous necrosis. Then, this amount was administered either alone or after conditioning with 5-HT. Under these circumstances, some of the metallic salts, e.g., $ZnCl_2$ (Groups 10—13), $MnCl_2$ (Groups 14—17), and lead acetate (Groups 18—20) exerted a more or less significant necrotizing action when administered in combination with 5-HT; other salts such as $CaCl_2$ (Groups 21,22), $InCl_3$ (Groups 23,24), and $KMnO_4$ (Groups 25—27), the polysaccharides carrageenin and agar (Groups 28—31) as well as lycopodium (Groups 32, 33), exhibited no sign of a latent necrotizing effect that could have been made manifest by 5-HT. Finally, ox bile, papain, and trypsin (Groups 34—43) proved to be excellent challengers for the ACN since after conditioning they produced intense necrosis when administered at otherwise well tolerated dose levels.

Even these preliminary data sufficed to show that hypertonicity is not an indispensable prerequisite of agents capable of challenging for the ACN. Apparently the phenomenon of conditioning is of importance in determining tissue resistance to a variety of seemingly unrelated agents.

In order to learn more about the nature of this response, a second series of experiments was performed in which some of the previously identified, as well as several additional, potential challengers were tested comparatively when given by themselves or after conditioning with 5-HT

Table 1. *List of challengers*

Group	Challenger*	Conditioner	Necrosis (mm)
1	5-HT 1 mg	5-HT 2 mg s.c.	0
2	5-HT 1 mg	Polymyxin 2 mg s.c.	0
3	5-HT 1 mg	Dextrin 250 mg i.v.	0
4	Polymyxin 1 mg	5-HT 2 mg s.c.	0
5	Polymyxin 1 mg	Polymyxin 2 mg s.c.	0
6	Polymyxin 1 mg	Dextrin 250 mg i.v.	0
7	Histamine 5 mg	5-HT 2 mg s.c.	0
8	Histamine 5 mg	Polymyxin 2 mg s.c.	0
9	Histamine 5 mg	Dextrin 250 mg i.v.	0
10	$ZnCl_2$ 5 mg	None	0
11	$ZnCl_2$ 5 mg	5-HT 2 mg s.c.	13 ± 4.8
12	$ZnCl_2$ 5 mg	None	8 ± 2.4
13	$ZnCl_2$ 5 mg	5-HT 2 mg s.c.	25 ± 1.7
14	$MnCl_2$ 6 mg	None	0
15	$MnCl_2$ 6 mg	5-HT 2 mg s.c.	1 ± 0.8
16	$MnCl_2$ 6 mg	None	26 ± 5.2
17	$MnCl_2$ 6 mg	5-HT 2 mg s.c.	14 ± 7.8
18	Lead acetate 50 mg	None	8 ± 4.5
19	Lead acetate 50 mg	5-HT 0.1 mg i.v.	3 ± 3.0
20	Lead acetate 50 mg	5-HT 2 mg s.c.	24 ± 4.7
21	$CaCl_2$ 10 mg	None	2 ± 1.4
22	$CaCl_2$ 10 mg	5-HT 2 mg s.c.	8 ± 2.9
23	$InCl_3$ 8 mg	None	0
24	$InCl_3$ 8 mg	5-HT 2 mg s.c.	4 ± 2.0
25	$KMnO_4$ 30 mg	None	0
26	$KMnO_4$ 30 mg	5-HT 0.1 mg i.v.	0
27	$KMnO_4$ 30 mg	5-HT 2 mg s.c.	0
28	Cgn 50 mg	None	0
29	Cgn 50 mg	5-HT 2 mg s.c.	0
30	Agar 50 mg	None	0
31	Agar 50 mg	5-HT 2 mg s.c.	0
32	Lycopodium 50 mg	None	0
33	Lycopodium 50 mg	5-HT 2 mg s.c.	0
34	Ox-bile 3%	None	0
35	Ox-bile 3%	5-HT 0.1 mg i.v.	0
36	Ox-bile 3%	5-HT 2 mg s.c.	34 ± 2.18
37	Papain 30 mg	None	0
38	Papain 30 mg	5-HT 0.1 mg i.v.	13 ± 4.9
39	Papain 30 mg	5-HT 2 mg s.c.	43 ± 3.1
40	Papain 30 mg	None	0
41	Papain 30 mg	5-HT 2 mg s.c.	20 ± 3.4
42	Trypsin 4 mg	None	0
43	Trypsin 4 mg	5-HT 2 mg s.c.	23 ± 2.9

* All challengers were injected in 2 ml of distilled water into the deep connective-tissue layer under the shaved skin of the back.

Table 2. *List of challengers*

Group	Challenger*	Conditioner	Necrosis (mm)
1	Glucose 1500 mg	None	0
2	Glucose 1500 mg	5-HT 2 mg s.c.	43 ± 1.3
3	Glucose 1500 mg	Polymyxin 2 mg s.c.	39 ± 2.1
4	Glucose 1500 mg	Dextrin 60 mg i.v.	35 ± 1.7
5	$CuCl_2$ 1 mg	None	0
6	$CuCl_2$ 1 mg	5-HT 2 mg s.c.	17 ± 3.0
7	$CuCl_2$ 1 mg	Polymyxin 2 mg s.c.	12 ± 3.3
8	$CuCl_2$ 1 mg	Dextrin 60 mg i.v.	10 ± 2.5
9	Na-metaphosphate 80 mg	None	4 ± 3.0
10	Na-metaphosphate 80 mg	5-HT 2 mg s.c.	38 ± 1.2
11	Na-metaphosphate 80 mg	Polymyxin 2 mg s.c.	23 ± 5.4
12	Na-metaphosphate 80 mg	Dextrin 60 mg i.v.	23 ± 1.4
13	$ZnCl_2$ 10 mg	None	8 ± 3.5
14	$ZnCl_2$ 10 mg	5-HT 2 mg s.c.	32 ± 1.5
15	$ZnCl_2$ 10 mg	Polymyxin 2 mg s.c.	29 ± 1.6
16	$ZnCl_2$ 10 mg	Dextrin 60 mg i.v.	28 ± 1.0
17	Formalin 0.5%	None	0
18	Formalin 0.5%	5-HT 2 mg s.c.	18 ± 7.6
19	Formalin 0.5%	Polymyxin 2 mg s.c.	(2 ± 2.0)
20	Formalin 0.5%	Dextrin 60 mg i.v.	21 ± 3.4
21	Acetic acid 1%	None	0
22	Acetic acid 1%	5-HT 2 mg s.c.	33 ± 0.7
23	Acetic acid 1%	Polymyxin 2 mg s.c.	33 ± 2.7
24	Acetic acid 1%	Dextrin 60 mg i.v.	32 ± 0.9
25	Ethanol 25%	None	0
26	Ethanol 25%	5-HT 2 mg s.c.	36 ± 0.2
27	Ethanol 25%	Polymyxin 2 mg s.c.	36 ± 1.5
28	Ethanol 25%	Dextrin 60 mg i.v.	33 ± 2.9
29	Tannic acid 30 mg	None	0
30	Tannic acid 30 mg	5-HT 2 mg s.c.	15 ± 9.7
31	Tannic acid 30 mg	Polymyxin 2 mg s.c.	8 ± 4.0
32	Tannic acid 30 mg	Dextrin 60 mg i.v.	(0)
33	Croton oil 3%	None	6 ± 1.9
34	Croton oil 3%	5-HT 2 mg s.c.	15 ± 2.8
35	Croton oil 3%	Polymyxin 2 mg s.c.	9 ± 2.0
36	Croton oil 3%	Dextrin 60 mg i.v.	6 ± 3.2
37	Bile 40 mg	None	0
38	Bile 40 mg	5-HT 2 mg s.c.	29 ± 0.7
39	Bile 40 mg	Polymyxin 2 mg s.c.	25 ± 1.1
40	Bile 40 mg	Dextrin 60 mg i.v.	0
41	Papain 25 mg	None	0
42	Papain 25 mg	5-HT 2 mg s.c.	48 ± 3.3
43	Papain 25 mg	Polymyxin 2 mg s.c.	34 ± 4.3
44	Papain 25 mg	Dextrin 60 mg i.v.	45 ± 7.1

Table 2 (continued)

Group	Challenger*	Conditioner	Necrosis (mm)
45	Trypsin 6 mg	None	0
46	Trypsin 6 mg	5-HT 2 mg s.c.	22 ± 3.5
47	Trypsin 6 mg	Polymyxin 2 mg s.c.	27 ± 2.4
48	Trypsin 6 mg	Dextrin 60 mg i.v.	19 ± 4.2
49	$CaCl_2$ 20 mg	None	20 ± 1.1
50	$CaCl_2$ 20 mg	5-HT 2 mg s.c.	6 ± 2.6
51	$CaCl_2$ 20 mg	Polymyxin 2 mg s.c.	8 ± 3.6
52	$CaCl_2$ 20 mg	Dextrin 60 mg i.v.	13 ± 3.4

* All challengers were injected in 2 ml of distilled water into the deep connective-tissue layer under the shaved skin of the back.

and with two types of mast-cell dischargers: polymyxin and dextrin. It will be recalled that dextrin produces an intense generalized mast-cell discharge with anaphylactoid inflammation only upon intravenous injection while polymyxin exhibits this effect even if given subcutaneously.

For control purposes we used hypertonic glucose solution which had previously been shown to be a powerful challenger for the ACN. This observation was confirmed, although here we used a somewhat smaller amount of glucose than in earlier work. It will not be necessary to comment on each of our series since sensitization by the conditioning agents was essentially the same with most of the challengers used. All three conditioning agents sensitized the tissues for the production of necroses by the various challengers; the few apparent exceptions were presumably due to premature mortality. (In these cases the readings in the column "Necrosis" are placed in brackets.) However, the challenging potency of the compounds tested was by no means equivalent. Thus the effect of croton oil—even when given at a dose which in itself produces some necrosis—was not significantly aggravated by conjoint treatment with any of the three conditioning agents.

In general the three conditioning agents tested here were essentially equivalent in their ability to sensitize for the production of necroses by any one challenger, yet one singular exception deserves comment. In the case of challenge by bile, dextrin exhibited no conditioning potency while 5-HT and polymyxin were efficacious. At first we thought that some technical error may have vitiated the results but the experiment was repeated twice with the same results. We have no explanation for this unusual finding but it suggests that all conditioners are not equivalent in their ability to sensitize for different challengers.

It is also noteworthy that the appearance of the necrotic patches was not always the same. While most challengers produced necrotic changes

essentially similar to those elicited by hypertonic solutions, the lesions produced by Na-metaphosphate were intensely hemorrhaged while those induced by acetic acid distinguished themselves by their extreme pallor, particularly sharp demarcation and unusually rapid development, being quite evident within 15 to 20 min.

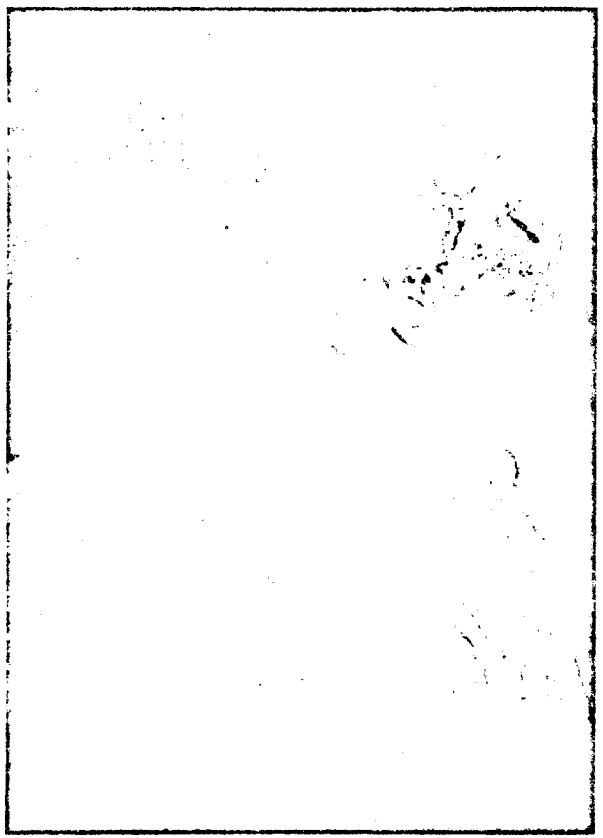

Fig. 1. *Acute necrosis elicited by papain following conditioning by vasopressin.* Top: Subcutaneous injection of 25 mg of papain on the back between the shoulder blades produces no detectable lesion in itself. Bottom: The same papain treatment after conditioning with 8 mg of vasopressin (given subcutaneously in the pubic region) leads to intense liquefaction necrosis at the site of papain injection. In addition, the dissolved papain-containing connective tissue has made a subcutaneous channel, descending by gravity to the abdominal surface where the liquefied material accumulated and eventually perforated to the outside. The observation clearly shows that, in addition to mast-cell dischargers and mast-cell products, vasopressin can also act as a potent conditioner for the ACN effect of an enzyme preparation

The groups challenged with $CaCl_2$ deserves special comment since in these, conditioning by any of the three agents actually inhibited the necrosis but greatly augmented the local calcification. The singular effect of

5-HT, polymyxin, and dextrin upon the $CaCl_2$-induced lesions will be the subject of a special study in view of its possible relationship to calciphylaxis and calcergy. Suffice it to point out here that evidently under certain circumstances, conditioning may also protect tissues against acute necrosis.

Discussion

Following conditioning with mast-cell dischargers or 5-HT, a number of compounds, essentially different from each other in their chemical structure and pharmacologic activity, can act as challengers for the ACN phenomenon. Although mast-cell dischargers and mast-cell products are among the most potent conditioners for the ACN, these compounds proved to be devoid of challenging activity. Apart from hypertonic solutions the most efficacious challengers found so far are: acetic acid, bile, papain, and trypsin.

It is difficult to find any common denominator between these potent challengers or compounds devoid of this activity. Perhaps the most essential characteristic of potent challengers is that they exert a very intense but evanescent damaging effect upon the tissues. Long-acting inflammatory irritants (croton oil, carrageenin, agar and many of the metallic salts) exhibit little, if any, challenging potency. Conversely such agents as acetic acid, ethanol, bile, and enzymes (whose histotoxicity is considerable but rapidly vanishing as a consequence of local inactivation or absorption) are the most efficacious. The pharmacologic actions of the mast-cell dischargers and mast-cell products are likewise short-lived; hence, they could not be expected to exert a pronounced influence upon the evolution of very long-lasting stimuli. In other words, the action-curve of the conditioners must roughly coincide in time with that of the challenger in order to make a pharmacologic interaction consequential. In this respect, sensitization for the ACN phenomenon is essentially different from the conditioning action of pro- and anti-inflammatory hormones, whose effect is slower to develop but much more prolonged and hence capable of affecting chronic inflammatory processes (Selye, 1953, 1954).

It is not yet known what role, if any, the ACN phenomenon may play in physiology and pathology. It may be taken for granted, however, that the body's reactivity to a great variety of potential tissue irritants can be decisively influenced by mast-cell products. This has been demonstrated in the present series of experiments by the induction of necrotic reactivity to such diverse agents as metallic salts, organic acids, bile, and proteolytic enzymes. It may be pertinent to point out here that according to earlier observations a single injection of 5-HT can also produce widespread muscle necrosis in vascular territories "challenged" by partial obstruction of their arterial blood supply (Selye, 1965).

For the sake of simplicity we always referred to the ACN-lesions simply as "necrosis", since this is their salient common characteristic; it is noteworthy however that the quality of the lesions varies considerably depending upon the nature of the challenger. Thus, for example, challenge by acetic acid produces anemic coagulation necrosis with little edema and no hemorrhage, while challenge by Na-metaphosphate results in the formation of intensely hemorrhagic edematous plaques. Bile, trypsin, and papain cause liquefaction necrosis with spreading lesions which usually sink through the connective tissue from the back to the belly where they eventually perforate the skin; on the other hand, after challenge by $CaCl_2$, there is intense topical calcification presumably because the injected calcium precipitates with endogenous phosphate and carbonate. Furthermore, we have often observed that when very small doses of challengers are administered, they fail to cause necrosis but instead elicit simple inflammation upon treatment with conditioners. It is evident therefore that although the ACN always depends upon the conjoint action of certain conditioners and challengers, it can be responsible for a great variety of phenomenologically quite distinct morbid lesions.

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Relation of Dietary Acetic and Butyric Acids to Intake, Digestibility, Lactation Performance, and Ruminal and Blood Levels of Certain Metabolites [1]

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Abstract

The hypothesis was tested that acetic and butyric acids are related to the lower dry matter intake and superior efficiency which usually results from feeding lactating cows silage rations compared to hay.

Voluntary intake of dry matter was not affected by 2% acetic, 1% butyric, or a combination of these acids added to a hay-concentrate ration. However, a mixture of 4% acetic and 2% butyric acids appeared to cause nasal irritation and reduced intake. Milk yield and milk fat content were not appreciably affected by treatments. Although acetic acid increased energy digestibility, none of the treatments improved efficiency of energy utilization. Rumen concentration of acetic and butyric acids was higher, and propionic acid lower, for the supplemented rations. Blood acetic and butyric acids were elevated by the acetic and the acetic-butyric acid supplements. These results generally did not support the hypothesis that acetic and butyric acids increased efficiency of dry matter utilization.

Voluntary consumption of dry matter from well-fermented silage is generally lower than that for hay from the same source. However, cows fed these forages usually produce similar daily yields of fat-corrected milk (FCM), resulting in superior efficiency in utilization of dry matter from the silage rations. The factor(s) responsible for the poor consumption of silage and its more efficient utilization have not been determined.

Since a major difference between hay and silage dry matter is the content of fermentation products, various of these substances have been examined in previous studies for effects on intake. Intraruminal administration of silage effluent (26) and dietary supplementation with various combinations of silage acids and acid salts in short-term preliminary studies gave variable results, but tended to support the idea that these acids may be involved.

Reduction in forage intake following intravenous administration of acetic acid also implicated silage acids in appetite regulation (8). However, others (5, 14) reported a positive relationship between blood levels of acetic acid and dry matter intake.

Lactation responses to administration of acetic and butyric acids have been variable. Ruminally infused acetic acid increased milk yield and fat percentage (19), as did dietary calcium acetate (28). Inclusion of sodium acetate in the ration at 2.9% had no effect on milk yield, whereas a level of 5.6% increased yields (23).

Effects of dietary butyric acid on milk yield have apparently not been tested. Zelter (28) reported that calcium butyrate had no effect on milk yield, but decreased milk fat content. However, calcium acetate plus calcium butyrate consistently increased yields of milk and fat. Ruminal infusion of butyric acid had no influence on milk yield, but increased the milk fat content (19). In contrast, Storry and Rook (25) found a depression in milk production following large ruminal infusions of butyric acid.

Negative correlations with net production efficiency have been reported (7, 9, 10) for ruminal acetic and the acetic-to-propionic acid ratio. The molar ratio of butyric acid in total rumen VFA was positively correlated with the requirement of TDN for milk production (10).

The effect of dietary acetic and butyric acids on lactation efficiency has not been established. This experiment was designed to examine the hypotheses that the lower intake and superior efficiency of silage dry matter for lactating cows may be related to its content of acetic and butyric acids. The effects of dietary acetic and butyric acids on ruminal and blood levels of various organic acids, glucose, and ketones were also investigated.

Experimental Procedure

One Guernsey cow and four Jersey cows were used in a 5 × 5 Latin-square design with a 2-wk preliminary period preceding the ex-

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TABLE 1

Rations used in the experiment

Ration component	Rations				
	C	A	B	LAB	HAB
	(%)				
Concentrate[a]	50.0	49.0	49.5	48.5	47.0
Alfalfa hay[b]	50.0	49.0	49.5	48.5	47.0
Acetic acid	.....	2.0	.....	2.0	4.0
Butyric acid	.....	.....	1.0	1.0	2.0

[a] The concentrate mixture contained 14.2% crude protein and was composed of nine parts ground corn, five parts ground oats, four parts wheat bran, and two parts soybean oil meal.
[b] Contained 17.9% crude protein and 22.3% crude fiber.

perimental periods. Each experimental period consisted of a 3-wk transition period and a 1-wk comparison period.

The mean chemical composition and gross energy contents of the hay and concentrate are shown in Table 1, and ingredients of rations used are listed in Table 2. The control ration (C) was composed of 50% concentrate and 50% chopped alfalfa hay. Composition of rations used is given in Table 1. Treatments were: 2% acetic acid (A), 1% butyric acid (B), 2% acetic + 1% butyric acids (LAB), and 4% acetic + 2% butyric acids (HAB) added as ration diluents in each case.

Each cow received the assigned ration twice daily at 6:30 AM and 6:30 PM. The acids were diluted 1:5 with water and one-third of this diluted acid was mixed at feeding time with the concentrate and two-thirds with the roughage portion of the ration. Steamed bonemeal and trace-mineralized salt were provided free choice and water was available continuously via automatic waterers. The feeds fed and refused were weighed at each feeding.

At the end of each experimental period, the daily samples of concentrate mixture and alfalfa hay were composited. Oven-air-dried samples were prepared from these composites and used for chemical analyses and energy determinations (6). Proximate analyses of the feed samples were made by AOAC methods (2). Lignin was determined by the procedure of Van Soest (27), and caloric values obtained with a Parr bomb calorimeter.

Digestibility of dry matter and feed nutrients were determined with the lignin-ratio method and calculations made using the formula of Balch (3). Grab samples of approximately 250 g feces were taken rectally and placed in polyethylene bags. Collections were made at 7:30 AM and 5:30 PM during each day of the comparison period. These samples were kept frozen and composited at the end of each experimental period. Gross energy, lignin, and proximate analyses were determined by the methods described previously for feed samples. Crude protein determinations were made on wet feces.

Milk samples were taken as aliquots of the milk produced at each milking during the comparison period. Milk fat percentage was determined by the Babcock method, the per cent solids-not-fat (SNF) by the Golding bead test.

TABLE 2

Dry matter consumption and apparent digestibility[a]

Rations	DM consumption	Digestibility					
		Dry matter	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract	Gross energy
	(Kg/day)	(%)					
Control[b]	16.0 a	58.1 a	67.8 a	52.3 a	36.2 ab	65.3 a	56.8 a
+ 2% acetic acid	16.1 a	61.5 a	70.4 a	56.5 a	38.7 a	68.5 a	61.4 b
+ 1% butyric acid	15.7 ab	58.7 a	67.7 a	50.6 a	35.9 b	66.4 a	57.2 a
+ 2% acetic + 1% butyric acids	15.2 ab	58.4 a	67.6 a	53.9 a	37.0 ab	65.2 a	59.3 a
+ 4% acetic + 2% butyric acids	14.1 b	58.8 a	67.6 a	53.3 a	37.8 ab	65.7 a	60.2 a

[a] Values with common letters are not significantly different ($P < .05$).
[b] Fifty per cent alfalfa hay, 50% concentrate mixture of nine parts ground corn, five parts ground oats, four parts wheat bran, and two parts SBOM.

and protein by the AOAC method (2). Gross energy of milk was measured by calorimetry, as described above, using 500–700-mg samples of dried milk. The dried milk was obtained by lyophilization.

Milk yield comparisons were based on the total production for the seven days in the comparison period. Milk production efficiency was calculated by the following formula: Milk production efficiency =

$$\frac{\text{DE consumed (Meal/day)} - \text{DE for maintenance}^3 \text{ (Meal/day)}}{\text{FCM (kg/day)}}$$

Cows were weighed following the evening milking on three successive days, at the beginning of each three-week experimental period and at the completion of the experiment.

Rumen fluid samples were taken via an esophogeal tube at 4 and 6 hr after feeding. Blood samples were obtained from the jugular vein at 10 and 12 hr following feeding. Rumen fluid and blood samples were analyzed for VFA, ketones, and glucose according to methods previously described (21).

Results and Discussion

Dry matter consumption. Consumption data presented in Table 2 show that only the high level of acetic-butyric acid mixture significantly ($P < .05$) depressed intake. Odors resulting from this high acid treatment were considerably more pungent than usually observed for even highly acid silage. Cows were noted to react in a manner suggesting repugnance, possibly from olfactory irritation. None of the other treatments appreciably reduced dry matter intake. This indicates that levels of acetic and butyric acids usually contained in silages do not depress intake sufficiently to account for differences frequently reported in consumption of hay and silage.

One hypothesis has been advanced that acetic acid acts as a chemostatic regulator of appetite by stimulating the satiety center(s) (8, 16, 22). However, in our present and previous study (21), as well as in experiments of Little and Hawkins (14) and Baumgardt et al. (5), higher intake levels were associated with elevation in blood acetic acid. Possibly, blood levels of the magnitude to cause appetite depression from acetic acid occur only with dietary levels above those found in common silages.

Apparent digestibility. Apparent digestibility was not greatly affected by treatments (Table 2). However, acetic acid as a single supplement tended to improve digestibility of several proximate constituents, but only gross energy digestibility was increased significantly ($P < .05$). Therefore, this difference appeared to arise from the cumulative effect of the slightly higher digestibility of all the major components. This difference was not associated with variations in feed intake. From the results it appears that acetic acid per se may have a beneficial effect on digestion.

Combination of acetic and butyric acids (both low and high levels) also resulted in slightly higher gross energy digestibility than the control ration. Dry matter, crude protein, and nitrogen-free extract digestibilities were similar for all rations, except the acetic acid–supplemented ration. Digestibilities were somewhat higher for the latter ration. Crude fiber digestion was lowest in the ration containing butyric acid as a single additive. Digestibility values for gross energy, dry matter, nitrogen-free extract, and protein were found to differ significantly ($P < .05$) among cows.

Milk production and efficiency. Mean data for actual and FCM production, solids-not-fat (SNF), milk protein, and fat percentage are shown in Table 3. No significant effects on any of these production criteria resulted from the acid supplements. Lack of response from acetic acid addition is in agreement with several reports (15, 23), but not with others (4, 19, 28). Stimulation by acetic acid of increased milk yield in certain experiments may arise from direct utilization by the mammary gland of acetic acid for lactogenesis. Failure of acetic acid to stimulate lactation in certain other studies may have been the result of adequacy of the basal ration in terms of quality of the substrate for ample acetic acid production in the rumen. For example, the ration fed by Zolter (28) may have been deficient in this respect, due to use of sugar beets and, consequently, a response in milk yield resulted from supplemental acetic acid. Lack of response could have resulted also from feeding above required total energy needs or from insufficient supplemental acetic acid (relative to total nutrients fed) to elicit a response. Rook and Balch (19), using relatively low-producing cows (8.2–14.5 kg/day), produced increased milk yields with 900 ml of acetic acid daily. In another study (4), higher milk yields resulted from feeding rations containing 558 g acetate daily. The amount of acetic acid provided in our rations averaged 600 ml per day for cows of considerably higher production.

Butyric acid tended to decrease actual milk yield, but had no appreciable effect on FCM. Similar results were reported previously (19,

[3] Reference (17).

TABLE 3

Lactation performance[a]

Rations	Actual milk	FCM	SNF	Milk protein	Milk fat	Efficiency[b]
	(kg/day)			(%)		(Meal)
C	17.1	18.7 a	9.8 a	4.1 a	4.8 a	1.51 a
A	17.0	17.9 a	10.0 a	3.9 ab	4.4 a	1.67 a
B	16.8	18.1 a	9.7 a	4.0 a	4.5 a	1.54 a
LAB	17.4	18.7 a	9.6 a	4.0 a	4.6 a	1.53 a
HAB	16.4	17.9 a	9.7 a	3.8 b	4.7 a	1.58 a

[a] Values with common letters are not significantly different ($P<.05$).

[b] Meal DE/kg FCM = $\frac{\text{DE consumed} - \text{DE for maintenance}}{\text{Milk yield}}$.

25, 28). Butyric acid per se probably has little, if any, direct effect on lactation, because of its extensive metabolism in the rumen wall and liver (1).

Although generally classified as lipotrophic, acetic acid did not improve the milk fat content in this study. The same factors discussed above concerning the effect of acetic acid on milk yield are probably applicable in explaining the effects on fat test. The basal ration used in the present experiment supported a relatively normal milk fat percentage for these cows. Others found that addition of acetate (400–1,500 g per day) to rations supporting normal fat tests did not improve milk fat percentage (4, 15). However, infusion of 900–1,500 ml acetic acid into the rumen (19), or feeding 400–1,500 g acetate to cows having a low milk fat percentage appreciably improved milk fat content (4, 23, 28).

Butyric acid addition had no apparent effect on milk composition. Although butyric acid is recognized as a precursor of lactose and protein (11, 13), effects of its addition to normal rations apparently have not been previously studied. The lactation performance in our study agrees well with the findings of Zelter (28), in which butyrate was fed as the calcium salt. In contrast, in other studies (19, 25), intraruminal infusion of butyric acid produced increases in milk fat as well as S.N.F.

The digestible energy available per kilogram of milk, in excess of theoretical maintenance needs, was lower for the ration containing acetic acid. This ration also resulted in a somewhat higher concentration and molar per cent of acetic acid in the rumen. Although these differences were not significant at $P < .05$, they tend to support results of Elliot and Loosli (9), Coppock et al. (7), and Hinders and Owen (10), who found negative correlations between the molar per cent of ruminal acetic acid and milk production efficiency.

These results suggest that the superior efficiency of ration dry matter utilization usually noted with silages compared with hays is likely caused by factors other than the acetic and butyric acid content of silages.

Blood ketones and glucose. Blood glucose and ketone values are summarized in Table 4. Mean ketone levels remained in the normal range for all treatments (2.57–5.84 mg/100 ml). At the 12-hr sampling time the HAB treatment produced significantly ($P < .05$) higher ketones than other treatments. None of the other values at 10 or 12 hr post-feeding differed significantly. Ketones for all treatments were higher at 12 hr than at 10 hr; whereas, glucose values were

TABLE 4

Blood ketone bodies and glucose[a]

Rations	Ketones		Glucose	
	10 hr	12 hr	10 hr	12 hr
	(mg/100 ml)			
C	2.93 a	3.27 a	39.65 a	37.27 a
A	2.57 a	3.49 a	37.67 a	36.55 a
B	3.37 a	3.51 a	38.52 a	38.87 a
LAB	3.02 a	3.66 a	36.65 a	35.75 a
HAB	3.89 a	5.84 b	38.98 a	34.65 a

[a] Values with common letters are not significantly different ($P < .05$).

generally higher ($P < .05$) at 10 hr than at 12 hr. Similar results were reported by Schultz and Smith (20) and Storry and Rook (24). A significant ($P < .05$) period effect on ketone levels was observed. Ketones increased from July through November, then declined.

In contrast to the ketone levels, blood glucose tended to be lower for the treatments than for the control ration. However, at neither 10 nor 12 hr after feeding did levels differ significantly among treatments. Treatment means ranged from 34.6 to 39.6 mg/100 ml.

Rumen and blood volatile fatty acids. Rumen VFA data are given in Table 5. Differences among rations were observed only at the 4-hr sampling time. Although the concentration of acetic acid in the rumen was slightly higher when the acetic acid supplement was fed, none of the ruminal VFA were significantly affected.

Supplemental butyric acid and the high level of acetic-butyric acids produced a higher ($P < .05$) butyrate level in the rumen than did the acetic acid ration. The high level of acids in combination significantly reduced propionic acid concentration. Otherwise, ruminal VFA were not appreciably affected by rations. The acetate-to-propionate ratio was higher for rations containing acetic and acetic plus butyric acids than for the other rations.

Blood VFA concentrations (Table 6) did not differ at 10 hr post-feeding. However, at 12 hr both acetic and butyric acid levels were significantly ($P < .05$) elevated by the A and HAB treatments. Butyric acid produced no detectable changes in blood VFA levels. These observations are in agreement with those of Storry and Rook (24) and Jorgensen et al. (12), and support the idea that acetic acid passes through the rumen wall with little or no change, whereas butyric acid is rapidly metabolized by rumen epithelial tissue (18).

TABLE 5
Ration effects on rumen VFA[a]

	Rations											
	C		A		B		LAB		HAB			
	4 hr	6 hr	4 hr	6 hr	4 hr	6 hr	4 hr	6 hr	4 hr	6 hr		
Acetic acid (A) (μM/ml)	83.3 a	70.2 a	89.7 a	72.9 a	83.0 a	77.4 a	83.7 a	80.2 a	80.1 a	74.0 a		
Propionic acid (P) (μM/ml)	23.6 a	20.3 a	22.1 a	18.8 a	22.9 a	20.2 a	20.0 a	19.1 a	17.0 b	17.3 a		
Butyric acid (B) (μM/ml)	9.0 ab	7.6 a	8.3 a	8.6 a	10.7 b	8.8 a	9.6 ab	8.7 a	10.3 b	8.8 a		
Isovalerie acid (μM/ml)	0.99 a	0.70 a	0.88 a	0.66 a	0.94 a	0.69 a	0.80 a	0.49 a	0.77 a	0.56 a		
Valeric (μM/ml)	0.80 a	0.73 a	0.68 a	0.68 a	0.67 a	0.57 a	0.59 a	0.71 a	0.60 a	0.62 a		
Acetic/propionic	3.57 a	3.46 a	4.07 a	3.87 a	3.63 a	3.83 a	4.20 ab	4.20 a	4.82 b	4.28 a		

[a] Values with common letters are not significantly different ($P < .05$).

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TABLE 6

Blood volatile fatty acids[a]

Rations	Acetic acid[a]		Propionic acid[a]		Butyric acid[a]		Valeric acid[a]	
	10 hr	12 hr	10 hr	12 hr	10 hr	12 hr	10 hr	12 hr
	(μM/ml)							
C	1.24 a	2.26 b	0.11 a	0.11 a	0.05 a	0.04 c	0.10	0.17
A	2.03 a	2.60 a	0.10 a	0.10 a	0.06 a	0.10 a	0.17	0.19
B	2.14 a	2.25 b	0.13 a	0.11 a	0.05 a	0.06 bc	0.17	0.22
LAB	2.59 a	2.80 a	0.12 a	0.17 a	0.07 a	0.04 c	0.20	0.14
HAB	2.27 a	2.58 a	0.16 a	0.15 a	0.08 a	0.07 b	0.14	0.24

[a] Values with common letters are not significantly different ($P < .05$).
[b] Not analyzed statistically.

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Quantitative Determination of Formic, Acetic, Propionic, and Butyric Acids by Gas Chromatography

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Volatile acids were separated from food samples by a steam distillation according to AOAC methods, neutralized, and evaporated to dryness. The acids were then liberated with an equivalent amount of 0.5*N* dichloroacetic acid in acetone. Three microliters of the acetone solution containing from 0.1 to 1.0 mg of each acid per ml was injected directly into the column of the gas chromatograph. Experimental conditions were: Six-foot glass column; support, Anakrom 110/120 mesh; substrate, 10.5% ethyleneglycol adipate and 1.75% phosphoric acid; column temperature, 100°C; Sr^{90} detector temperature, 170°C; vaporizer temperature, 200°C; detector voltage, 1400 volts; argon flow rate, 50 ml per minute.

Peak heights and responses relative to methyl enanthate (heptanoate) as an internal standard were used to calculate concentrations of the individual acids. Recoveries from known mixtures were 95–105% for lower concentrations of the acids and 97–101% for higher concentrations. Results of analyses of frozen whole eggs and of frozen fish fillets agreed with results by AOAC methods.

Volatile acids are important as indexes of decomposition in many foods and as constituents of food flavors. The characteristic increase of the volatile acids content during decomposition of foods provides a means for classifying the different stages of the decomposition process (1, 2). Separation, identification, and quantitation of the volatile acids also offers valuable information on the composition of food flavors.

According to AOAC methods (3), volatile acids are isolated from food products by steam distillation. Acetic acid and the higher homologs are separated by column chromatography and determined by titration of the eluted fractions. Formic acid is analyzed separately by reaction with mercuric chloride. The application of quantitative gas chromatography was investigated for the following advantages: A single procedure for all of the volatile acids, improved specificity and sensitivity, and greater speed. For regulatory purposes, the chromatograms should provide useful confirmation of the results obtained by the AOAC methods.

Experimental

This paper outlines the method which was adopted and the several approaches taken in developing it. During the development of this method the gas chromatography of the free acids and of their methyl, ethyl, and benzyl esters was explored. The methyl and ethyl esters were easily chromatographed on column supports coated with various polyesters at temperatures below 100°. However, quantitative recovery of the esters from the esterification mixture was difficult because of their high volatility and appreciable solubility in water. In addition, methyl alcohol and ethyl alcohol interfered with the chromatographic separation because the retention times of the alcohols were similar to those of the corresponding esters. Molecular sieves were used for removing ethyl alcohol, but with limited success. These unique problems are not encountered in the gas chromatography of methyl esters of the higher fatty acids which have high boiling points and are insoluble in water. Alcohol and volatile solvents can be removed without appreciable loss of these esters. In order to work with higher boiling esters, benzyl esters of C_1 to C_4 acids were prepared and chromatographed on polyester columns at 175°. The chromatograms were satisfactory, but incomplete esterification and the formation of polymeric byproducts limited the usefulness of this approach.

Because of the difficulties encountered with esterification procedures, methods involving gas chromatography of the free acids were investigated. Ether solutions of the C_1 to C_4 acids were chromatographed on Chromosorb column support coated with dimer acid-silicone oil 550. The instrument used was a Perkin-Elmer Model 154-B Fractometer equipped with filament detector, metal injection port, and metal column. Figure 1 shows that the formic acid was completely lost. Similar results were obtained with other column substrates, each containing a small percentage of nonvolatile organic acid. A Research Specialties gas chromatograph equipped with ionization detector, metal injection port, and metal column was also used. Sensitivity was improved, but the formic acid could not be detected, and the chromatograms were similar to Fig. 1. The metal system probably catalyzed the decomposition of formic acid to carbon dioxide and hydrogen.

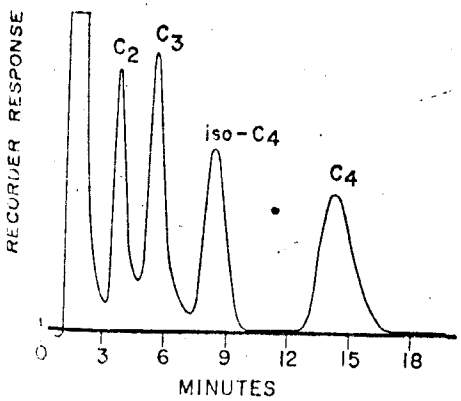

Fig. 1—Chromatogram of C_2–C_4 Acids. C_2, Acetic; C_3, Propionic; Iso-C_4, Isobutyric; and C_4, Butyric Acid.
Instrument: Perkin Elmer 154B, Katharometer Detector. Current: 280 ma; Column 6' × ¼", 25% Dimer Acid-Silicone Oil 550 on Chromosorb W, 60 mesh. Carrier Gas: Helium; Flow Rate: 20 ml/min.; Chart Speed: 20 inch/hr; Attenuation: X8; Column Temp.: 128°C.

The metal injection port of the Fractometer was replaced with the Perkin-Elmer glass dipper injection system. Figure 2 shows that formic acid was separated and detected in a mixture of C_1 to C_5 acids which contained equal amounts of formic and acetic acids. However, the small peak for formic acid demonstrates that the recovery of formic acid was not complete. The protection afforded by the glass injection system at the point of highest temperature accounted for the partial recovery of formic acid.

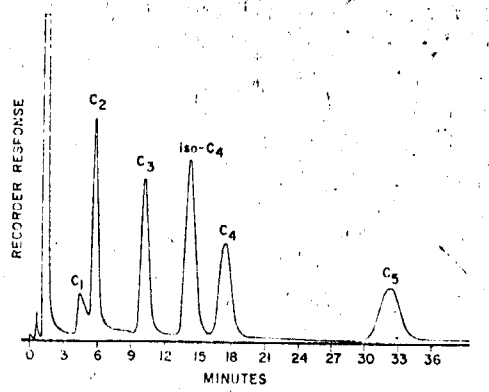

Fig. 2—Chromatogram of C_1–C_5 Acids. C_1, Formic; C_2, Acetic; C_3, Propionic; C_4, Butyric; C_5, Valeric Acid.
Instrument: Perkin Elmer 154B, Katharometer Detector, with glass microdipper injection system. Current: 290 ma; Column: 6' × ¼", 25% Didecylphthalate, 2% Sebacic Acid on Chromosorb W, 60 mesh. Carrier Gas: Helium; Flow Rate: 40 ml/min.; Chart Speed: 20 inch/hr; Attenuation X32; Column Temp.: 136°C.

Because of the small percentages of volatile acids encountered in our food decomposition studies, it was necessary to have greater sensitivity than that provided by the filament detector. Since hydrogen-flame detectors do not respond to formic acid, the work was continued with a Research Specialties gas chromatograph equipped with a strontium-90 detector. It was necessary to modify the instrument in order to have the advantage of an all-glass injection and column system. The instrument was originally equipped with a horizontal metal injection port leading into a 6' stainless steel column by means of ⅛" stainless steel tubing. The metal housing at the top of the column oven was replaced by a modified housing which accommodated a Barber-Colman type 6' glass column. The flash heater was disconnected from the metal injection port, connected firmly to the injection zone of the glass column, and supported by the metal housing.

The gas connections were rearranged to permit bypassing the metal injection port and injecting directly into the column. Formic acid and the other acids were then chromatographed satisfactorily.

Hunter, *et al.* (1) reported a method for the gas chromatography of free acids from acetic to capric acid on a diethyleneglycol adipate column. They started with the sodium salts and liberated the acids with dichloroacetic acid in acetone. The free acids were soluble in acetone but the sodium dichloroacetate was insoluble. Liberation of the acids in a nonaqueous medium and simplicity of operation were distinct advantages of their procedure. It was therefore adopted in principle, but for our application it was necessary to make the following changes: (*a*) The concentration of dichloroacetic acid was reduced from 7*N* to 0.5*N* in order to permit more accurate acidification of the sodium salts. By avoiding a large excess of dichloroacetic acid, the stability of the final solution was improved. (*b*) Phosphoric acid was included in the column substrate to achieve quantitative recovery of formic acid as well as of other volatile acids. (*c*) An internal standard or marker of known concentration was added to each solution as a reference for calculating acid concentrations. (*d*) The column temperature was lowered from 120° to 100° to insure good resolution of the peaks for the solvent, internal standard, formic acid, and acetic acid.

Figure 3 shows a chromatogram obtained with a Carbowax-phosphoric acid column. The order of peak emergence was acetic, propionic, formic, and butyric acids. Iso-butyric acid had the same retention time as propionic acid. A normal order of emergence and better resolution were obtained with a column coated with ethyleneglycol adipate-phosphoric acid (Figs. 4 and 5).

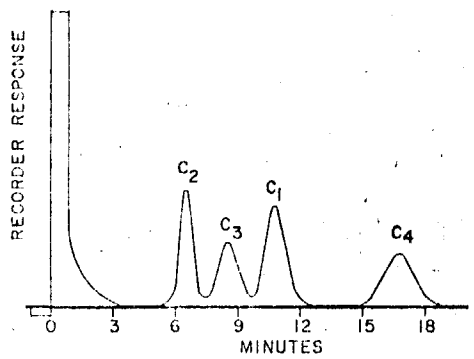

Fig. 3—Chromatogram of C_1–C_4 Acids. C_2, Acetic; C_3, Propionic; C_1, Formic; and C_4, Butyric Acid. Instrument: Modified Research Specialties Gas Chromatograph, Ionization Detector Sr-90. High Voltage: 1400 volts. Column 6′ × ¼″, glass; 12% Carbowax 1540, 5% H_3PO_4 on Anakrom A, 100 mesh. Carrier Gas: Argon; Flow Rate: 65 ml/min.; Chart Speed: 30 inch/hr; Attenuation: X10; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 103°C.

METHOD

Reagents

(a) *Acetone.*—Redistilled.

(b) *Dichloroacetic acid (DCA) solution (0.5N).*—Redistill and weigh 0.615 g DCA into a 10 ml volumetric flask, and dilute to 10 ml with acetone. Prepare daily.

(c) *Methyl enanthate.*—Weigh 250 ml anhydrous methanol in a 500 ml flask, cool in an icebath, and transfer to hood. Bubble BF_3 slowly through a glass tube into the methanol until 31.3 g BF_3 is taken up. BF_3 must be passing through the glass tube before the tube is placed in methanol (5). Weigh 7 g enanthic acid (heptanoic acid) (b.p. 223°) into an erlenmeyer and add 70 ml of the 12.5% by weight BF_3-methanol solution. Boil 3 minutes on steam bath. Cool, and transfer to a 750 ml separator containing 210 ml H_2O and 140 ml petroleum ether. Shake 3 minutes. Remove lower layer. Add 10 ml 5% Na_2CO_3 solution to separator and shake. Remove lower layer. Add 20 ml H_2O to separator, shake, and draw off H_2O; repeat once. Filter petroleum ether extracts through funnel with glass wool plug covered with Na_2SO_4 into erlenmeyer. Evaporate the solvent to 40 ml on steam bath; complete solvent removal in N stream. Distill residual methyl enanthate, collecting the fraction boiling at 172–172.5°. Store in tightly stoppered glass vial in refrigerator.

(d) *Methyl enanthate (ME) internal standard solution.*—Dilute about 0.35 g methyl enanthate, weighed to nearest mg, to 100 ml with acetone.

(e) *Dilute methyl enanthate (dil. ME) internal standard.*—Dilute 1 ml ME internal standard solution to 10 ml with acetone.

(f) *Standard acid solutions.*—Formic, acetic, propionic, and butyric acids, 0.05*N*. Check purity of stock solutions of acids initially by gas chromatography. Prepare acid dilutions for calibration by pipeting the calculated volume of each acid into a separate 200 ml volumetric flask (0.39 ml formic, 0.57 ml acetic, 0.75 ml propionic, and 0.92 ml butyric acid). Dilute to mark with acetone. Pipet a 20 ml aliquot of acid dilution into an erlenmeyer, add 20 ml water and 2 drops phenolphthalein, and determine the acid content by titrating with 0.05*N* NaOH.

$$\text{mg acid/ml standard soln} = \text{ml } 0.05N \text{ NaOH} \times F/20,$$

where F = weight of acid equivalent to 1 ml 0.05*N* NaOH: 2.30 mg formic, 3.00 mg acetic, 3.70 mg propionic, and 4.40 mg butyric acid.

Apparatus

(a) *Gas chromatograph.*—Use gas chromatograph equipped with an argon ionization detector and an all-glass injection and column system.

Parameters for modified Research Specialties gas chromatograph:

Column temperature 100°, detector temperature 170°, injection zone temperature 200°, argon flow rate 50 ml/min., high voltage 1400 volts, attenuation × 5, and 6′ glass column.

Optimum conditions for the gas chromatographic separation are obtained when the peaks for solvent, internal standard, and formic acid are completely resolved. Standard and acid peaks are sharp, although formic and acetic acid peaks are not completely resolved. Conditions vary to some degree from instrument to instrument and should be experimentally re-established.

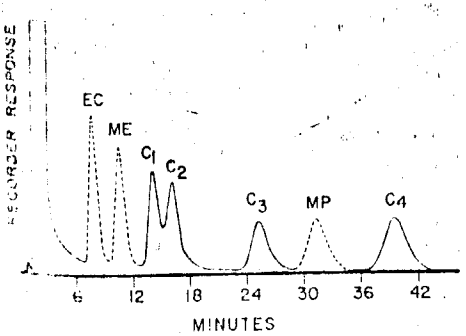

Fig. 4—Chromatogram of C_1–C_4 Acids and Internal Standards. C_1, Formic; C_2, Acetic; C_3, Propionic; C_4, Butyric Acid; EC (Ethyl Caproate), ME (Methyl Enanthate) and MP (Methyl Pelargonate). Instrument: Modified Research Specialties Gas Chromatograph, Ionization Detector Sr-90. High Voltage: 1500 volts. Column: 6′ × ¼″, glass, 10.5% Ethylene Glycol Adipate, 1.75% H_3PO_4 on Anakron ABS, 80–120 mesh. Carrier Gas: Argon; Flow Rate: 50 ml/min.; Chart Speed: 20 inch/hr; Attenuation: X5; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 100°C.

(b) *Preparation of column packing.*—Weigh 1.3 g of ethylene glycol adipate (Applied Science Laboratories, State College, Pa.), into a 150 ml beaker and dissolve by stirring in 70 ml acetone. Into second beaker weigh 0.26 g phosphoric acid and dissolve in 30 ml acetone. Combine both solutions. Weigh 12.5 g Anakrom ABS (Analabs) into 500 ml round-bottom flask, add combined acetone solutions, and evaporate in a rotating evaporator at reduced pressure in water bath at 35° (will pack one 6′ column).

Calibration

Pipet into same 5 ml volumetric flask a volume of each standard acid solution and a volume of ME internal standard, as below:

Individual Acid Concn in Sample, mg	*C_1–C_4 Acid Std Soln (0.05N), ml*	*Vol of Internal Std Added, ml*	*Concn[a]*
less than 1	0.25–0.5	1.0	(c)
1.0–2.5	0.5 –1.0	0.5	(d)
2.0–5.0	1.0 –2.0	1.0	(d)

[a] See *Reagents*.

Dilute to mark with acetone and mix. Repeat, using varying volumes of acids to provide a range of concentration for each acid (see Table 1). Inject 3 µl aliquots into gas chromatograph. Measure peak heights on chromatograms and calculate R value for each acid:

$R_A = h_A c_{ME}/c_A h_{ME}$, where h_A and h_{ME} are the peak heights of the acid and methyl enanthate, respectively, and c_A and c_{ME} are the corresponding concentrations (mg per 5 ml acetone solution).

Determination

Quantitatively neutralize the steam distillate of the volatile acids obtained by AOAC method 18.019 with 0.01*N* NaOH and phenolphthalein as indicator. Add a known excess of base (1 ml 0.1*N* NaOH). If color is discharged, repeat addition. Concentrate to about 45 ml on steam bath in air stream. (If color is discharged, add 0.5 ml more 0.1*N* NaOH), transfer the solution to a 50 ml beaker, and evaporate to dryness in air stream. Loosen the salts with a micro spatula and transfer carefully through a funnel (1.5 cm diameter) into

tube-type, glass-stoppered volumetric flask (see table, last column, for volume).

0.01N NaOH for Neutralization of Steam Distillate (Reduce Addn of Excess), ml	Volatile Acid Content as Acetic Acid, mg	Vol. of Internal Std Added, ml	Concn[b]	Total Vol. After Dilu, ml
1.0–1.5	0.6–0.9	1.0 (dil)	(c)	2.0
1.5–3.0	0.9–1.8	0.5	(d)	5.0
3.0–6.0	1.8–3.6	1.0	(d)	5.0
6.0–8.0	3.6–4.8	1.0	(d)	5.0
9.0–12.0	5.4–7.2	2.0	(d)	10.0

[a] For fish samples, add 1 ml ME internal standard; for egg samples, add only 0.5 ml (because the volatile acids are more evenly distributed between formic and acetic acid).
[b] See *Reagents*.

Add to beaker, rinsing walls, volume of 0.5*N* DCA equivalent to total NaOH added to distillate. Transfer through funnel into the volumetric flask. Repeat the rinsing with 0.5 ml or more acetone, and add to volumetric flask. Swirl the flask to disperse the salts and to liberate the volatile acids. Pipet a volume of dilute ME standard or ME standard (as specified for acid content of sample in table above) to volumetric flask and mix. Centrifuge 3 min. at low speed in the glass-stoppered tube to separate the precipitated sodium dichloroacetate. Inject 3 μl of clear solution in gas chromatograph. Measure peak heights and calculate acid concentrations with R values established in calibration. $c_A = h_A c_{ME}/R_A h_{ME}$.

To calculate mg of each acid in weight of sample used for distillation, divide by fraction recovered in distillation. Typical recoveries for first 200 ml distillate are: Formic acid 0.405, acetic acid 0.57, propionic acid 0.81, and butyric acid 0.92 (6).

Results and Discussion

Internal Standard.—In order to obtain quantitative results it was necessary to correct for variations in volume of sample injected, and for variations in the sensitivity of the detector from run to run. With known amount of marker compound or internal standard added to each acetone solution of acids it was possible to correct for such variations.

Figure 4 shows the positions of three esters which can be used as standards: Ethyl caproate (EC), methyl enanthate (ME) and methyl pelargonate (MP). Methyl octanoate interferes with the acetic acid peak. Because of the emphasis on formic and acetic acids as primary indexes of decomposition in certain foods, methyl enanthate was the preferred standard. Methyl pelargonate may have some advantage if butyric acid is the acid of principal interest.

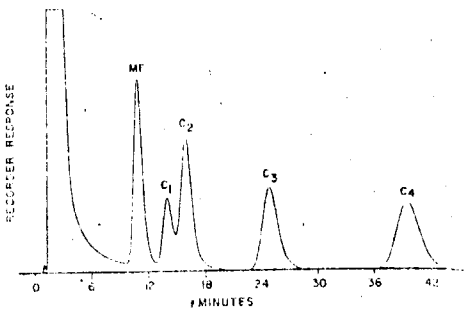

Fig. 5—Chromatogram of C_1–C_4 Acids and Methyl Enanthate. ME (Methyl Enanthate), C_1, Formic; C_2, Acetic; C_3, Propionic; and C_4, Butyric Acid. Instrument: Modified Research Specialties Gas Chromatograph, Ionization Detector Sr-90. High Voltage: 1400 volts. Column: 6' × ¼", glass, 10.5% Ethylene Glycol Adipate, 1.75% H_3PO_4 on Anakrom ABS, 110/120 mesh. Carrier Gas: Argon; Flow Rate: 50 ml/min.; Chart Speed: 20 inch/hr; Attenuation: X5; Detector Oven Temp.: 170°C; Injection Zone Temp.: 200°C; Column Temp.: 100°C.

Table 1. Response values of volatile acids relative to methyl enanthate

Range of Acid Concn in Sample, mg	R Value: Low	High	Range	Average R Value
Formic Acid				
0.204–1.633	0.668	0.723	0.055	0.678
1.633–2.449	0.668	0.675	0.007	0.671
2.449–4.496	0.672	0.675	0.003	0.673
Acetic Acid				
0.615–1.230	0.633	0.674	0.041	0.659
1.230–2.460	0.633	0.645	0.012	0.641
2.460–4.200	0.645	0.652	0.007	0.648
Propionic Acid				
0.645–1.289	0.326	0.342	0.016	0.338
1.289–2.578	0.326	0.337	0.011	0.335
2.578–4.921	0.337	0.339	0.002	0.338
Butyric Acid				
2.368–4.700	0.285	0.291	0.006	0.289

Calibration.—Bartlet and Smith (7) described a method for correcting peak heights of partially resolved peaks. Their method assumes a Gaussian distribution and estimates the peak-height correction from the peak separation in units of σ. In our chromatograms (Figs. 4 and 5) the distance between the formic acid and acetic acid peaks was approximately 2.8 σ, which corresponded to a peak-height correction of approximately 2%. This correction was disregarded; it was compensated by the internal standard.

The detector response (S) is defined as the peak height (h) per unit concentration. Concentration (c) is expressed in terms of mg of compound in total volume of the acetone solution. For the methyl enanthate standard (ME),

$$S_{ME} = h_{ME}/c_{ME} \qquad (1)$$

For each volatile acid (A) in the mixture of standard acids,

$$S_A = h_A/c_A \qquad (2)$$

For each acid, relative response (R) is defined as

$$R_A = S_A/S_{ME} = (h_A \times c_{ME})/(c_A \times h_{ME}) \qquad (3)$$

Having established R values for each acid, unknown concentrations were calculated by substituting the R values in equation 3:

$$c_A = (h_A \times c_{ME})/(R_A \times h_{ME}) \qquad (4)$$

Table 2. Recovery of volatile acids

Acid	mg in Sample	Equivalent mg/100 g Food: Fish AOAC 18.015	Liquid Egg AOAC 16.035	mg Found	% Recovery
Formic	0.204	2.02	1.47	0.214	105
Acetic	0.615	1.32	3.15	0.646	105
Propionic	0.645	3.18	2.32	0.661	103
Formic	0.408	4.03	2.94	0.429	105
Acetic	0.615	4.32	3.15	0.581	95
Propionic	0.645	3.18	2.32	0.648	101
Formic	0.817	8.06	5.88	0.819	100
Acetic	1.230	8.63	6.30	1.258	102
Propionic	1.289	6.37	4.64	1.289	100
Formic	1.633	16.13	11.76	1.643	101
Acetic	2.460	17.26	12.59	2.501	102
Propionic	2.578	12.73	9.28	2.578	100
Butyric	2.368	10.29	7.50	2.273	96
Formic	2.218	22.20	16.18	2.212	98
Acetic	1.200	29.47	21.49	1.201	100
Propionic	1.921	24.30	17.72	1.921	100
Butyric	1.700	20.11	14.90	1.536	97
Formic	2.449	24.19	17.63	2.449	100
Acetic	2.460	17.26	12.59	2.462	100
Propionic	2.578	12.73	9.28	2.578	100
Formic	4.496	44.40	32.37	4.505	100
Acetic	1.200	29.47	21.49	1.238	101
Propionic	1.920	24.30	17.72	1.900	100

Table 3. Comparison of volatile acid determination by gas chromatography and by AOAC methods 16.305 and 18.015 (3)

Sample	AOAC	Gas Chromatography Individual Results[a]				Av.
Formic Acid, mg/100 g						
Egg						
1	0					trace
2	0	0.54	0.58	0.56	0.55	0.6
3	11.5	10.8	10.9	11.7	11.3	11.2
4	9.7	9.99	9.99	10.0		10.0
5	0	1.04	1.13	1.07	1.11	1.1
6	35.7	31.2	31.3	31.1	31.2	31.2
Fish						
1	0	0.66	0.70	0.73		0.7
2	0	0.68	0.63	0.63	0.72	0.7
3	trace	1.28	1.16	1.00		1.1
4	5.0	3.90	3.83	3.95	3.99	3.9
5	7.6	3.29	3.26			3.3
6	5.5	4.46	4.56	4.44		4.5
Acetic Acid, mg/100 g						
Egg						
1	0	1.64	1.63	1.65		1.6
2	0	2.03	2.03	2.06	2.04	2.0
3	19.0	18.2	18.2	18.4	18.3	18.3
4	15.7	15.4	15.7	15.7	16.3	15.8
5	9.4	7.17	6.92	7.01	7.17	7.1
6	37.8	34.6	35.7	35.0	34.6	34.8
Fish						
1	5.5	4.24	4.24	4.29	4.28	4.3
2	6.8	3.31	3.30	3.40	3.34	3.3
3	10.3	7.63	8.45	7.95	7.69	7.9
4	7.6	6.43	6.45	6.36	6.51	6.4
5	42.4	37.8	38.8	40.0	40.3	39.2
6	105	101	102	102	102	102.5

[a] Replicates of the same solution.

In equations *1* and *2*, a linear relationship between peak height and concentration was assumed for any single run, and any intercept correction at zero concentration was ignored. The relative response term (R_A) from equation *3* was a constant for each acid, and it corrected for differences in the response of the detector to the various acids. The reciprocal detector response for the standard (c_{ME}/h_{ME}) in equation *4* corrected for variations in volume of sample injected and for observed variations in instrument sensitivity between runs. Areas can be substituted for peak heights in equations *1* to *4*. However, with the internal standard method, peak heights proved to be satisfactory for calculating acid concentrations and were used for simplicity.

Results.—Table 1 shows the experimental *R* values for the C_1 to C_4 volatile acids over a wide range of concentrations. Each value is the average of six determinations. The largest variation (range 0.055, or 8% of the *R* value) occurred with the lowest concentration of acid. Errors in measuring peak heights and any intercept correction in equation *2* would be more important at low concentrations. The average value for each acid was nearly constant over the entire range of concentrations tested.

Table 2 shows recovery data for mixtures of acids of known concentration. The data are presented in increasing order of formic acid concentration. Columns 3 and 4 show the calculated equivalent concentrations of the acids if present in fish and in eggs. These values were derived from the figures in column 2 by applying the appropriate computations in AOAC methods **18.015** and **16.035** (3). The results in column 5 are the averages of 4 determinations on each sample. The recoveries were 95–105% for the lower concentrations of acids and 97–101% for the higher concentrations.

Samples of frozen whole egg and of frozen fish fillets were analyzed for volatile acids by the gas chromatographic method and by AOAC methods **16.035** and **18.015** (3). Table 3 shows the agreement in results by the two methods and also demonstrates the greater sensitivity of the gas chromatographic method for the lowest concentrations of acids.

Acknowledgment

The assistance of Cynthia Campbell in analyzing the fish and egg samples by AOAC methods is gratefully acknowledged.

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This paper was presented at the Seventy-sixth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 15–17, 1962, at Washington, D.C.

ITALIAN TRANSLATION

Minimum Lethal Distant Dose of Some Sodium Salts Injected Intravenously: Comparative Toxicity of Some Anions

Source: Bollettino della Societa Italiana di Biologia Sperimentale 14: 136-37, 1939.

The term minimum lethal distant dose by the intravenous route (which others designate as minimum lethal dose at a distance) is used by me to designate the smallest dose of a drug, which introduced into the veins at a concentration and a rate which in itself is not toxic, kills the animal after a certain period of time following the end of the injection. I shall not speak here about the great difference existing between the immediate minimum lethal dose and the distant minimum lethal dose by the intravenous route, referring this subject to my preceding studies (1). I only want to remind the reader that I was the first one to find and describe the curves (graphs) of immediate toxicity by the intravenous route (2), which were then recovered and described many years later by foreign authors and correctly attributed to me by Beccari (3).

Convinced for many years of the need of giving a definite aspect to the problem of lethal doses, which is considered by many authors as a most personal and least precise factor that can be imagined (due to the fact that an international unit of measurement has not yet been established), while it almost always constitutes the basis of our pharmacological tests, everytime that I have had a chance to do so at our institute, I have determined the minimum lethal distant dose of a wide variety of drugs. Thus, as a result of these studies, we owe our knowledge of a considerable number of such doses. It seems useful to me to report such doses of numerous sodium salts by listing them together in Table 1. Since the

cation is always the same and the less toxic of all, we thus have a perfectly reliable toxicity scale of many anions, since it was obtained by unambiguous methods and under completely identical conditions.

TABLE 1

Sali Salts		Dose minima letale lontana in g eq per kg (1)	Tossicità posta = 1 quella del NaCl (2)
Sodico solfato (3)	(4)	0,05650	1,13
» cloruro (4)	(5)	0,05000	1
» piruvato (5)	(6)	0,04300	0,86
» bromuro (6)	(l. c., 5)	0,03900	0,78
» gluconato (7)	(7)	0,03500	0,70
» nitrato (8)	(8)	0,03100	0,62
» monofosfato (9)	(9)	0,02800	0,56
» acetato (10)	(10)	0,02600	0,52
» joduro (11)	(l. c., 5)	0,01400	0,28
» (bi) fosfato (12)	(11)	0,00900	0,18
» (13) tartrato neutro	(l. c., 10)	0,00750	0,15
» (14) solfito	(12)	0,00550	0,11
» (15) fluoruro	(l. c., 5)	0,00250	0,05
» (16) persolfato	(l. c., 5)	0,00150	0,03
» (17) nitrito	(l. c., 8)	0,00050	0,01

(1) *Archivio di Scienze Biologiche*, 1927, 12, 478; *Archivio it. di Sc. Farmacol.*, 1933, 2, 425.
(2) *Bollettino delle Scienze Mediche di Bologna*, 1905, 5, (ser. 8), 74.
(3) *Archives it. de Pharmac. et de Thérapie*, 1938, 68, 437.
(4) Da Val E. - *Archivio it. di Scienze Farmacol.*, 1933, 2, 445.
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(6) Gajatto S. - (In corso di stampa). (in press)

1--Minimum lethal distant dose, in g equiv./kg
2--Toxicity, equal to 1, of NaCl
3--Sodium sulfate
4--Sodium chloride
5--Sodium pyruvate
6--Sodium bromide
7--Sodium gluconate
8--Sodium nitrate
9--Sodium monophosphate
10--Sodium acetate
11--Sodium iodide
12--Sodium diphosphate
13--Sodium neutral tartrate
14--Sodium sulfite
15--Sodium fluoride
16--Sodium persulfate
17--Sodium nitrite

These studies show that the sulfate (sulfite) anion is the least toxic anion, and the most toxic is the nitrate (nitrite) anion, which is 100 times more toxic than the chloride anion.

I believe that these experiments, and other similar ones that will be assembled as soon as possible, can be used as a basis for a unit of international comparison.

LA DOSE MINIMA LETALE LONTANA PER VIA ENDOVENOSA DI ALCUNI SALI DI SODIO: TOSSICITA' COMPARATA DI ALCUNI ANIONI. Di I. Simon.

Con la dizione *dose minima letale lontana per via endovenosa* (che altri chiama dose minima letale *a distanza*) intendo la dose più piccola di un farmaco che, introdotta nelle vene in concentrazione e con velocità per sè non tossiche, uccide l'animale dopo un certo tempo dalla fine dell'iniezione. Non mi fermo qui a parlare della diversità profonda che passa fra dose minima letale immediata e dose minima letale lontana per via endovenosa, rimandando per questo ai lavori miei precedenti (1). Solo voglio ricordare qui che io fui il primo a trovare ed a descrivere le curve di tossicità immediata per via endovenosa (2), ritrovate poi e descritte molti anni dopo da autori stranieri e giustamente rivendicate a me da Beccari (3).

Persuaso da molti anni della necessità di dare un assetto definitivo al problema delle dosi letali, che sono negli autori quanto di più personale e di meno preciso si possa oggi immaginare (dato che non si è ancora stabilita un'unità di misura internazionale) mentre costituiscono quasi sempre la base delle nostre esperienze farmacologiche, tutte le volte che in Istituto mi si porse il destro ho fatto determinare la dose minima letale lontana di svariatissimi farmaci. A così fatte ricerche debbo la conoscenza di un numero notevole di tali dosi. Mi pare utile riportare, raccogliendole insieme, nella tabella I, quelle di numerosi sali di sodio. Essendo sempre lo stesso il catione, il meno tossico fra tutti, abbiamo così una scala di tossicità di molti anioni, perfettamente attendibile, in quanto venne fatta con metodi univoci ed in condizioni sperimentali perfettamente uguali.

Tabella I.

Sali		Dose minima letale lontana in g eq per kg	Tossicità posta = 1 quella del NaCl
Sodico solfato	(4)	0,05650	1,13
» cloruro	(5)	0,05000	1
» piruvato	(6)	0,04300	0,86
» bromuro	(l. c., 5)	0,03900	0,78
» gluconato	(7)	0,03500	0,70
» nitrato	(8)	0,03100	0,62
» monofosfato	(9)	0,02800	0,56
» acetato	(10)	0,02600	0,52
» joduro	(l. c., 5)	0,01400	0,28
» (bi) fosfato	(11)	0,00900	0,18
» tartrato neutro	(l. c., 10)	0,00750	0,15
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(4) Da Val E. - *Archivio it. di Scienze Farmacol.*, 1933, 2, 445.
(5) Ravasini G. - *Ibidem*, 1933, 2, 428.
(6) Gajatto S. - (In corso di stampa).

Risulta da queste ricerche che il meno tossico fra gli anioni studiati è l'anione solforico, il più tossico il nitroso, che è 100 volte più tossico dell'anione cloro

Credo che queste esperienze ed altre analoghe, che raccoglierò quanto prima, possano servire come base ad un'unità di confronto internazionale.

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(Dall'Istituto di Farmacologia della R. Università di Pisa).
Sezione di Pisa - Seduta del 23 gennaio 1939-XVII.

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J. Food Sci. 36(1):36-38, (1971)

STABILIZATION OF CARROT JUICE BY DILUTE ACID TREATMENT

SUMMARY--A problem arises if juice extracted from raw carrots is heated to about 180°F before or during conventional canning practices. An unsightly, unappetizing coagulum forms and the color of the juice precipitates with the coagulum. The coagulum is not as evident if the carrots are heated 5 min in water prior to juice extraction, but the juice loses some of its bright-orange color. The canned juice extracted from carrots heated 5 min in a .05 N acetic acid solution does not coagulate and the amount of juice which can be extracted from the carrots is 3.3% greater than that from carrots heated 5 min in water. The centrifuged juice from acid-treated carrots had a brighter orange color than juice from the water-treated carrots. The color notation was Rd, 23.7, a, 30.4 and b, 34.8 for juice from acid-treated carrots compared with a notation of Rd, 19.2, a, 24.4 and b, 31.4 for the juice from water-treated carrots. The canned juice extracted from raw carrots had less pectic substances and starch and about the same amount of protein as the canned juice from the carrots heated in water or .05 N acetic acid, but the canned juice from raw carrots coagulated. The juice from carrots heated in acid does not form a coagulum and maintains its orange color, probably because of the action of the heat and acid on the protoplasmic liquid of the cell before or immediately after the cell is ruptured.

INTRODUCTION

IT IS difficult to extract carrot juice from the vegetable tissue and heat it to a sterilizing temperature without undesirable changes taking place in the juice. The high temperature required for sterilizing raw carrot juice causes a coagulum to form in the juice. A smaller amount of coagulum will form if the raw carrots are heated in water before juice extraction. Tressler and Joslyn (1961) reported that in one operation whole carrots were blanched at 200°F for 5 min, ground and the extracted juice acidified with citric acid. One of the products was adjusted to about pH 4.2 and another with a pH of 5.28 was not adjusted. Both juices had a characteristic sweet, clean, carrot flavor.

Several investigators have recommended other methods of preparing canned carrot juice. Lachele (1938) prepared a carrot juice resumbling orange juice in color and consistency by passing carrots through a Schwarz comminution machine. The machine is capable of extracting 60-80% of the juice from raw carrots. The juice is heated at 180°F to coagulate unstable material, then passed through the comminution machine for homogenization, which prevents coagulation during further heat treatment. Further treatment consists of filling the juice hot into 8-oz cans, exhausting to 160°F and processing for 30 min at 240°F.

Beattie and Pederson (1943) ground carrots and pressed them in a hydraulic press, but the product as well as the yield was unsatisfactory. However, a satisfactory yield was obtained from a R.Y.P. extractor or from a Chisholm-Ryder continuous press. They found that carrots were most difficult to extract with a satisfactory yield of juice, and very fine maceration before pressing was necessary.

Another method of preparing carrot juice is described by Turner (1939). The carrots are blanched 15 min in boiling water, crushed and the juice extracted in a hydraulic press. From the press, the juice flows through 2 screens to a storage tank, then to the filling machines. Filled cans are exhausted to 185°F, sealed, cooked 22 min at 240°F and cooled as quickly as possible to 100°F.

Marsh (1942), Cruess et al. (1937) and Beattie and Pederson (1943) added acid or juice from acid-type vegetables to low-acid vegetable juice after extracting the juices from the vegetable tissue; they reported the acidified juices to be more palatable and easier to process than unaltered juices.

The previous investigators did not mention treating the vegetables with acid prior to grinding and juice extraction. It was the purpose of this investigation to study the effect on the canned juice and presscake of carrots heated in weak acid solutions before the carrots were ground and the juice extracted.

EXPERIMENTAL

IMPERATOR variety carrots in lots of approximately 200 lb each were obtained from the reject belt of a local packing-shed over a 2.5-month period. The lots of carrots were obtained March 1, 8 and 14, April 26 and May 1, 6, 13, 15 and 20. The carrots were washed and all insect-damaged and severely discolored ones removed. Duplicate 25-lb samples of the cleaned carrots were taken from the original 200-lb lot for juice extraction. Juice was extracted from raw carrots, carrots heated in water and those heated in a weak acid solution. The carrots were heated in 30 liters of distilled water or 30 liters of the weak acid solution, thoroughly drained and ground through a Fitzpatrick comminuting machine fitted with a .064-in. screen. The juice was extracted from the weighed ground carrots in a Palmer rack and cloth-type press. The pressure on the cake was increased slowly to 6000 psig and held at this pressure for 15 min. The juice was heated to 180°F in a steam kettle, filled hot into 303 × 406 enameled cans, sealed and processed 30 min at 240°F. The processed cans were cooled rapidly in tap water. Approximately 4-lb samples of the presscake were packaged in polyethylene bags and frozen in still air at −10°F.

25-lb samples of carrots were heated at several time intervals, using various concentrations of hydrochloric, citric and acetic acid. The extracted juice was tasted and a concentration of .05 N acetic acid arbitrarily selected as the heating media for the remainder of the tests. Carrots were heated in water for 0, 5, 15 and 25 min and in .05 N acetic acid for 0, 3, 5 and 15 min to determine heating time prior to juice extraction to obtain a desirable quality juice.

The percentage yield of juice, suspended solids, percent light transmission, °Brix, pH, titratable acidity and color were determined on juice from the 9 lots of carrots. The pectic substances, starch and protein were determined on the juice and presscake of the last 4 lots of carrots.

Table 1—Yield and analysis of canned juice from raw carrots and carrots heated in water and in acetic acid for increasing lengths of time.[a]

Minutes heated	Juice yield (%)	Light trans. (%)	Brix (°)	pH	Titratable acidity (%)	Color Rd	Color a	Color b
Juice from carrots heated in water								
0	74.0	95	7.2	6.1	.07	3.3	-3.0	7.5
5	71.3	59	7.2	5.7	.07	18.3	17.8	29.5
15	52.4	67	7.5	5.4	.09	16.2	12.5	28.9
25	48.9	74	7.0	5.3	.09	14.6	10.3	27.8
Juice from carrots heated in 0.5 N acetic acid								
0	74.0	95	7.6	6.0	.09	1.1	-2.1	5.4
3	75.4	32	8.6	5.5	.12	21.1	28.3	33.0
5	71.0	31	8.5	5.3	.13	25.1	33.7	35.2
15	54.7	36	8.2	5.1	.17	25.6	24.0	36.4

[a]Data represent the mean of duplicate determinations for each treatment, except that the 25-min cook in water and the 15-min cook in .05 N acetic acid represent 1 determination.

Suspended solids were determined by centrifuging 50 ml of juice 10 min at 1250 rpm according to the procedure recommended in the United States Standards for Grades of Grapefruit Juice (1968). The liquid was decanted from the top of the centrifuge tube and analyzed. The percent light transmission through the juices was determined by diluting 10 ml of the decanted liquid to 100 ml and reading on a Lumetron Colorimeter fitted with filter No. 580. Total titratable acidity was determined by titrating with standardized sodium hydroxide to an end point of pH 8.2. °Brix was measured on a Zeiss refractometer, pH measured with a Beckman Zeromatic pH meter and color determined with a Gardner Color Difference meter standardized with color plate MY-1: Rd, 27.3, a, −3.3 and b, 34.7.

Each can of juice was thoroughly mixed and a sample removed for determination of pectic substances, starch and protein. The frozen presscake was cut into pieces and samples weighed for pectic substances, starch and protein while the cake remained frozen. The pectic substance of the juice and presscake was extracted by the method of McColloch (1952) and pectic substances determined by the colorimetric method of Dietz and Rouse (1953). Starch was extracted from the samples of carrots according to the procedure of McCready et al (1950) and percent starch determined by the method of Roberts and Friloux (1965). Protein was determined according to the method in A.O.A.C. Official Methods of Analysis (1965). (Kjeldahl N × 6.25.) Data were subjected to an analysis of variance as described by Steel and Torrie (1960).

RESULTS & DISCUSSION

THE CANNED juice from carrots heated in a weak acetic acid solution prior to juice extraction was compared with the canned juice from carrots heated in boiling water and with canned juice from raw (unheated) carrots.

A .05 N acetic acid solution was decided upon as the heating media because a coagulum did not form and the canned juice retained its typical carrot flavor after processing. Neither did juice prepared from carrots heated in a .05 N hydrochloric acid solution coagulate during canning, but it was felt that use of a food-grade acid would be more acceptable, should the process be commercialized. Juice from carrots heated in a .05 N citric acid coagulated during canning. However, juice from carrots heated in a .1 N citric acid solution did not coagulate and could have been used. Those who tasted the juices considered the juice from carrots heated in the acetic acid solution to have the most typical carrot flavor.

The carrots were heated 5 min in water or acid because the combination of the highest yield and best quality of juice from the heated samples was obtained at this time interval (Table 1). As the heating interval in water or acid increased, the yield of juice decreased and the orange color of the juice faded. The juice from carrots heated 3 or 5 min in .05 N acetic acid was about the same in quality but, in order to compare the juice from carrots heated in water with the juice from carrots heated in acid, the 5-min heating interval was chosen.

The amount of juice extracted from the raw carrots was not significantly different from the juice extracted from the heated carrots (Table 2). There was an increase of 3.3% yield in juice from carrots heated in acid compared with juice from carrots heated in water, and the increase was highly significant. The amount of juice which could be extracted from each lot of carrots with the methods employed was approximately the same, whether the carrots were obtained from the packing-shed on March 1 or May 20. These data show that the maturity and condition of the cull carrots are rather uniform throughout the harvesting period. Early in the harvest period there may be a slightly higher percentage of small immature carrots delivered to the packing-shed than during the latter part of the harvest period, but the differences are not large enough to greatly influence juice yield from the cull carrots. A processor in South Texas who wishes to can carrot juice from cull Imperator carrots could expect the yield of juice to remain rather constant throughout the harvest period.

There is a decrease in suspended solids and percent light transmission and an increase in orange color intensity of juice prepared from heated carrots as compared with juice prepared from raw carrots (Table 2). The same relationship exists if juice prepared from carrots heated in acid is compared with juice from carrots heated in water. A heavy orange-colored coagulum formed in the canned juice prepared from raw carrots. The light transmitted through the transparent juice was 97% and the color was light-green with a color notation of Rd, 1.7, a, −3.6 and b, 4.5. This juice would not be acceptable as a canned carrot juice.

The 1.5% suspended solids in the juice from the water-treated carrots were not statistically different from the .9% suspended solids in the juice from acid-treated carrots, but the 2 juices were different in percent light transmitted through the juices and the color of the juices was different. The juice from water-treated carrots allowed 50.2% of the light to pass through the centrifuged juice, whereas the juice from the acid-treated carrots allowed only 35.1% of the light to pass through the centrifuged juice. The centrifuged juice from acid-treated carrots had a brighter orange color than the juice from water-treated carrots. The color notation was Rd, 23.7, a, 30.4 and b, 34.8 for juice from the acid-treated carrots compared with a notation of Rd, 19.2, a, 24.0 and b, 31.4 for the juice from water-treated carrots. These differences are due to differences in the composition of the material comprising the suspended solids of the 2 juices. Small particles of the ground carrots were squeezed through the press-cloth during the extraction of both juices and were washed into the container as the juices were collected from the press. The suspended solids of the juice from the acid-treated carrots was made up mostly of these small carrot pieces, whereas the small carrot pieces plus the coagulum comprised the suspended solids of the juice from carrots heated in water. The coagulum which formed and settled from the juice from water-treated carrots removed the suspended solids responsible for color in the juice.

The increase in titratable acidity and decrease in pH between juices of the 3 treatments is probably due to heat decomposition of the pectic substances to pectinic acid and the absorption of the acetic acid by the carrots during the acid treatment of the carrots.

The amounts of the pectic substances, starch and protein in the canned juice and presscake are presented in Table 3. The juice from raw carrots had less pectic substances and starch and about the same amount of protein as the 2 other juices but formed the most coagulum during the canning operation. The heavy coagulum

Table 2—Yield and analysis of canned juice from raw carrots and carrots heated in boiling water and in acetic acid.[a]

Sample treatment	Juice yield (%)	Suspended solids (%)	Light trans. (%)	Brix (°)	pH	Titratable acidity (%)	Color Rd	Color a	Color b
Raw	71.1	9.9	97.0	8.1	6.1	.08	1.7	-3.6	4.5
Heated in boiling water	68.4	1.5	50.2	8.5	5.7	.11	19.2	24.0	31.4
Heated in .05 N acetic acid	71.7	.9	35.1	8.9	5.1	.15	23.7	30.4	34.8
Raw vs. heated	NS	**	**	NS	**	**	**	**	**
Water vs. acid	**	NS	**	NS	**	**	**	**	**

**Significant at the 1% level.

[a]Data represent the mean of duplicate determinations on 9 replications.

Table 3—Analysis of canned juice and presscake from raw carrots and carrots heated in water and in acetic acid.[a,b]

Sample treatment	Canned juice: Pectic substances Water (%)	Oxalate (%)	Acid (%)	Total (%)	Starch (%)	Protein (%)	Press cake: Pectic substances Water (%)	Oxalate (%)	Acid (%)	Total (%)	Starch (%)	Protein (%)
Raw	.05	.09	.01	.15	.25	9.0	.77	7.39	9.45	17.61	8.96	6.2
Heated in boiling water	.40	.35	.02	.77	.40	7.6	1.19	6.67	8.61	16.47	8.23	8.5
Heated in .05 N acetic acid	.28	.15	.02	.45	.33	7.8	.87	7.02	9.73	17.62	7.67	8.1
Raw vs. heated	**	**	NS	**	**	NS	NS	*	NS	*	**	**
Water vs. acid	**	**	NS	**	NS	NS	**	NS	**	**	NS	NS

*Significant at the 5% level; **Significant at the 1% level.
[a]Data represent the mean of duplicate determinations on 4 replications.
[b]Expressed as % dry wt.

which occurred in the canned juice from raw carrots was thought to have been caused by the heat denaturation of the protein. The coagulated protein precipitated from the juice and in the process took the .15% total pectic substances and the .25% starch down with it.

The coagulum which occurred in the juice extracted from carrots heated in water was also believed to be due to the denaturing of the protein. However, the heating process caused more of the pectic substances and starch to be extracted into this juice than was extracted into the juice from raw carrots. The additional heating during the canning process caused partial coagulation as it did in the juice from raw carrots, but the increase in pectic substances and starch acted as a stabilizer, to hold most of the coagulum in suspension. Kertesz (1951) pointed out that the pectic substances play an important part in the stabilization of the colloidal systems in fruit juices. It is reasonable to believe the same system would help stabilize carrot juice.

Another possible explanation for the partial stabilization of the juice from heated carrots is that the easily denatured proteins coagulated and remained in the presscake, whereas different and less heat-sensitive proteins were extracted into the juice. Additional heating at a higher processing temperature was either insufficient to cause the less heat-labile proteins to coagulate or the pectic substances and starch held most of them in suspension; consequently, only a small precipitate occurred in the juice.

The data in Table 3 would seem to indicate that the juice from carrots heated in acid should contain more coagulum than the juice from carrots heated in water, because there are less pectic substances, less starch and the same amount of protein in the juice from acid-treated carrots compared with juice from water-treated carrots. However, in this juice there is a 2-fold simultaneous treatment of heat and acid on the juice. The amount of protein is the same in this juice as in the juice from carrots heated in water, but the action of the acid has changed the proteins so that although there is less pectic substances and starch in the juice, there is a sufficient amount to keep any coagulated protein in suspension. Tressler and Joslyn (1961), in their discussion of juice from nonacid vegetables, state that when the vegetable is macerated, the enzymes released act upon the released protoplasmic liquid causing rapid metabolic changes unnatural to the undamaged tissues. The juice from carrots heated in acid does not form a coagulum and maintains its orange color because of the action of the heat and acid on the protoplasmic liquid of the cell before the cell is ruptured or immediately thereafter. Those materials responsible for changes in the juice, whether they are enzymes or denatured protein, or a combination of the two, are altered by the action of the heat and acid.

After the juice was extracted from carrots heated in acid, the remaining 28.3% presscake contained 8.1% protein. This by-product could be valuable as a food or feed supplement.

There is an estimated 20—24 thousand tons of carrots discarded each year in the South Texas area as cull or reject carrots, because the carrots are misshapen, too small, too large or have small blemishes as a result of growing and harvesting. A large percentage of these carrots are good food and could be manufactured into a very good juice product. Manufacturing these reject carrots into an edible product would greatly reduce the disposal problem often confronting the packer of fresh carrots.

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Effect of histamine and aspirin on healing of standardized gastric ulcers in dogs

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Several experimental models of chronic gastric ulcers[1-3,5,6] have been used to study both normal healing and the effect of selected medications upon normal healing. Many investigators[1,3,6] prefer the thermocauterization ulcer produced by burning the gastric mucosa. Using this technique, Skoryna and colleagues[3] produced ulcers averaging 6 mm. in diameter in the glandular portion of the rat stomach. These ulcers were studied by killing the experimental animals at selected intervals following various drug regimens in order to obtain photographic and histologic samples for subsequent correlation. Complete healing occurred within eight weeks and the pattern of healing appeared histologically similar to that which is seen in human peptic ulcer disease. The disadvantages of this technique are: (1) the need for a gastrotomy, which may alter normal ulcer development and subsequent healing, and (2) the inability to obtain serial observations during the healing phase in the same animal.

Takagi and co-workers[1] circumvented the need for gastrotomy in the production of gastric ulcers by transserosally injecting small amounts of acetic acid into the wall of the rat's stomach. They produced chronic ulcers which averaged 9 mm. in diameter and required up to six months to heal. The disadvantages of this technique are: (1) all measurements were made at postmortem examination, thereby precluding premortem serial observations; (2) all ulcers were made in the glandular portion of the rat stomach so that healing of similar ulcers in the nonglandular portion could not be compared.

The purpose of the present study was to: (1) evaluate the acetic acid ulcer in a different species, namely, the dog; (2) compare healing of this type of ulcer in the antrum and the body of the dog stomach; (3) obtain serial observations during the development and healing phases of these ulcers by means of twice weekly gastroscopy and gastric photography with the use of a fiberoptic gastroscope with camera*; and (4) compare the effects of aspirin and histamine upon normal healing of this acetic acid ulcer.

METHODS

Thirty-two dogs were anesthetized with intravenous pentobarbital with a dose of 28.5 mg. per kilogram of body weight. A midline laparotomy was performed and after the anterior portion of the stomach was exposed, 0.5 ml. of 10 percent acetic acid was injected locally (0.25 ml. submucosally and 0.25 ml. subserosally) through a 26 gauge needle into the anterior wall of both the body and the antrum of the stomach.

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*The gastroscope used in this study was the Olympus GTF-A.

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The stomach body injection was made 3 cm. proximal and to the left of the incisura angularis, whereas the antral injection was made about 7 cm. proximal to the pylorus and 1 cm. from the greater curvature to facilitate subsequent gastroscopic examination. Leakage of the acetic acid was prevented by applying digital pressure for three minutes at the site of injection following removal of the needle. Preliminary studies were made in 12 dogs to refine the technique and select a final dosage. Following injection, the serosa was covered by the greater omentum and the abdomen was closed. The total operative procedure required less than 30 minutes and was well tolerated by all the dogs.

All 32 dogs were examined by gastroscopy with a gastrocamera at two, five, and seven days, by which time the ulcers had reached maximal size. To ensure a clear field, the dogs were fasted for 15 hours prior to gastroscopy; these examinations were made while the animals were under general anesthesia. The gastroscope was protected from the teeth by a special mouth piece. Systematic visualization and photography of the stomach were then recorded. Over 60 gastroscopic examinations made on dogs have shown that estimates of ulcer size correlate with autopsy measurements to within an average of 1 mm. in diameter (standard deviation, 0.89 mm.) with the autopsy measurements being slightly smaller.

Three dogs died of gastric perforation within five days. The remaining 29 dogs were examined on the seventh day and then divided into three groups: (1) the control group comprised of 13 dogs that received no medications; four of the group were killed immediately to serve as a base line; (2) histamine group comprised of eight dogs that received a daily intramuscular injection of 2 mg. per kilogram of body weight of histamine in beeswax; (3) the aspirin group comprised of eight dogs that received a daily oral dose of 120 mg. per kilogram of body weight of commercial crystalline aspirin diluted in 500 c.c. drinking water, which was given in two daily aliquots and was well tolerated throughout the study period.

The dogs were then examined twice weekly with a fiberoptic gastroscope with camera. The last observation was made immediately prior to the killing of the dogs at three or six weeks following ulcer production. Repeat photographs and measurements of the fresh specimens were obtained at autopsy. The specimens were then fixed in formalin for histologic study with the use of hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) stains of both the ulcers and the nonulcerated mucosa. Serial blood histamine and serum salicylate levels were measured in each dog. The degree of PAS uptake in the superficial gastric mucosa was used as a semiquantitative index of mucosal mucin and over-all mucosal integrity. The ulcer size was recorded as an ulcer index (UI = length × width in millimeters) as the ulcer configuration varied from one dog to another.

RESULTS

Gastroscopic examination on the second day following ulcer production showed an area of round or elliptical mucosal swelling 1.2 cm. in diameter and, on occasion, minimal central hemorrhage at the point of previous injection. By the fifth day, the area of swelling had necrosed, producing an excavating ulcer 4 mm. in depth with sharp margins and an irregular black base. Marked mucosal undermining and swelling were present around this base. At seven days, the base had a more homogeneous black appearance and the UI averaged 154 mm. (Fig. 1, *A*). During the healing phase, the base became smoother and developed a white or pale fibrous lining, while the mucosal folds became less edematous and gradually converged toward the center of the base (Fig. 1, *B*). The UI at three weeks averaged 20. By six weeks, the ulcer had completely healed (UI = 0.0) with the mucosal folds radiating into a central depression covered by early regenerating

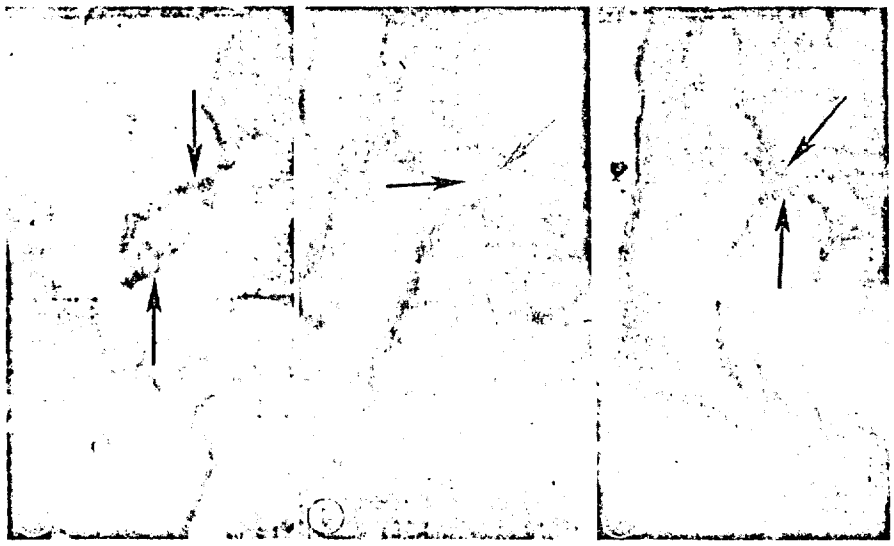

Fig. 1. Sequential healing of an acetic acid ulcer in the body of the stomach of a control dog. Antral ulcers were similar. See text. *A*, Healing at one week. *B*, Healing at three weeks. *C*, Healing at six weeks.

Table I. *Average ulcer index**

	Antrum		*Body*	
Group	*3 wk.*	*6 wk.*	*3 wk.*	*6 wk.*
Control	13	0	27	0
Histamine	60	36	68	25
Aspirin	15	0	35	26

*Ulcer index = length × width. Ulcer healing in both the body and the antrum of histamine-treated dogs was significantly delayed at three and six weeks (all p values < 0.001), whereas healing was significantly delayed in only the stomach body of the aspirin-treated dogs at six weeks ($p = 0.005$). PAS-positive mucosal staining was inversely related to ulcer healing for all groups.

Table II. *Mucosal erosions and PAS staining*

	Antrum		*Body*	
Group	*Erosions*	*PAS uptake**	*Erosions*	*PAS uptake**
Control (9 dogs)	None	2.6	None	2.8
Histamine (8 dogs)	Marked	0.5	Moderate	0.5
Aspirin (6 dogs)	None	2.6	Moderate	1.5

*PAS mucosal uptake was graded so that 0 = absent, 1 = minimal, 2 = moderate, and 3 = normal. Histamine erosions became more numerous during the experiment whereas this progression was not observed in aspirin-treated dogs.

mucosa (Fig. 1, *C*). The mucosa in nonulcerated areas appeared normal on each specimen throughout the six-week period of observation. Histologic changes of the antral and body ulcers are summarized in Fig. 2. The histologic appearance of both the antral and fundic mucosa in nonulcerated areas was normal with both H and E and PAS stains.

Histamine group. Most of the dogs receiving histamine appeared nauseated, and some regurgitated or defecated shortly after the histamine injection. Four dogs developed melena during the ensuing six weeks. Gastroscopic examinations were done in the morning prior to histamine injections in view of the excessive secretions which were subsequently stimulated. Histamine levels in blood, drawn approximately two hours after histamine injection, averaged 8 μg percent (range, 5.5 to 14.6). While gastroscopic examination showed that the rate of healing of ulcers in both the antrum and the body was significantly slower than those in the control group (Table I), the histologic appearance was identical. In contrast, marked differences were observed in the nonulcerated mucosa (Table II). By two weeks after the initiation of histamine injections, all

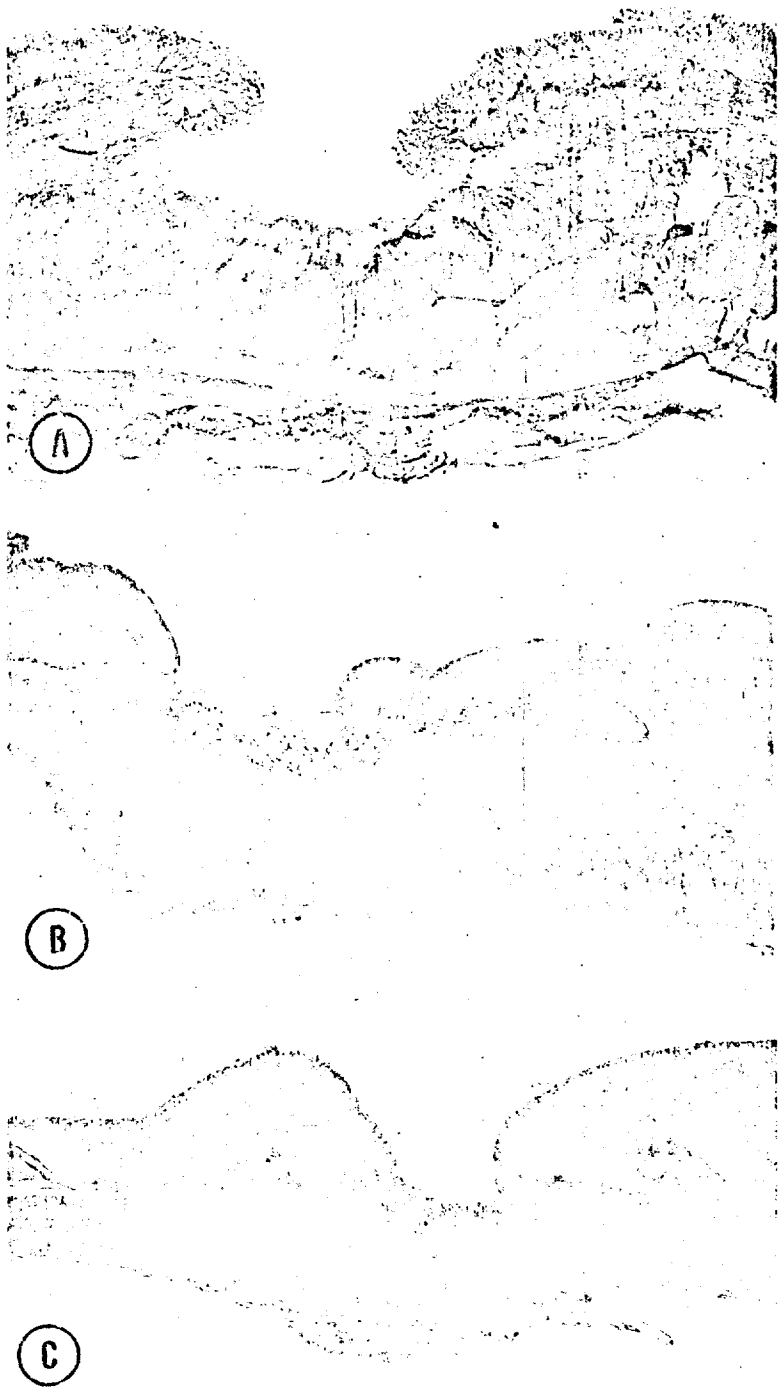

Fig. 2. Histologic appearance at one week (*A*) shows penetration through the muscularis which is replaced by a deep layer of granulation tissue and a superficial layer of necrotic debris. By three weeks (*B*), the base is smaller, granulation tissue is mature, and young regenerating mucosa is present along the edges. By six weeks (*C*), the base is covered by regenerated mucosa and is healed.

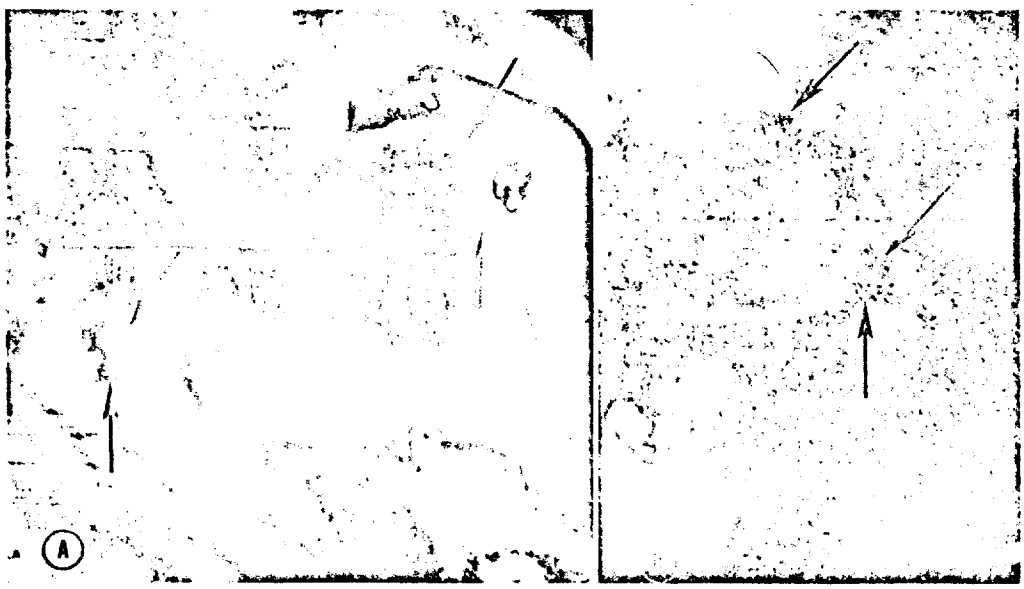

Fig. 3. Mucosal erosions were most striking in the antrum of the histamine-treated dogs *(A)* and were also significant in the stomach body of the aspirin-treated dogs *(B)*. See text.

eight dogs developed diffuse erosions measuring 1 to 7 mm. in diameter and 1 to 2 mm. in depth (Fig. 3, *A*). These erosions were more prominent in the antrum and became progressively worse during the subsequent six weeks and on several occasions were observed to be actively bleeding at the time of gastroscopy. Unlike the control group, PAS staining of the superficial mucosa cells was markedly decreased throughout the stomach but most strikingly in the antrum (Table II).

Aspirin group. Three dogs in the aspirin group exhibited mild anorexia but none vomited, developed melena, or had excessive gastric secretion during gastroscopy. The serum salicylate levels measured in the morning averaged 11.2 mg. percent (range, 8.4 to 17). The antral ulcers healed at the same rate as the control ulcers. The ulcers in the body of the stomach, however, showed significantly delayed healing (Table I). The histologic pattern was identical to that of the control ulcers. Within a week of aspirin administration, seven of the eight dogs developed black-based erosions measuring 1 to 8 mm. in diameter and up to 1 mm. in depth (Fig. 3, *B*). These were exclusively located in the body of the stomach where PAS mucosal staining was also decreased (Table II). This decrease in PAS staining was not as great as that seen in either the stomach body or antrum of those dogs in the histamine group (Table II).

DISCUSSION

The production of standardized gastric ulcers and the sequential observation of these without the need to open the stomach periodically or to kill the animal prematurely has previously hampered experimental design. An approach with the use of acetic acid for the former and the flexible fiberoptic gastroscope with camera for the latter has provided an opportunity to observe the dynamics of normal healing and healing against the background of aspirin and histamine administration. Gross and histologic postmortem examinations have confirmed the accuracy of these gastroscopic findings made immediately prior to death, and the ability to document on film the changing configuration of the surrounding gastric mucosa has been a very valuable research benefit.

Using this new approach, we were in-

terested in studying the effect of hyperacidity produced by histamine and mucin changes produced by chronically administered aspirin on the rate of healing of established standardized ulcers. On the basis of our findings, the response of antral ulcers associated with hyperacidity differed from those associated with aspirin administration in that hyperacidity retarded the rate of healing, whereas aspirin had no effect. In contrast, both histamine and aspirin significantly delayed healing of standardized ulcers created in the body. The explanation for this is not clear. Distinctive changes in the adjacent mucosa were striking, however, in that histamine greatly reduced the amount of mucin present throughout the stomach and was associated with marked erosions, especially in the antrum. The chronic administration of aspirin, on the other hand, produced virtually no change in the antrum but a moderate number of erosions in the body of the stomach and a significant diminution in the PAS mucosal uptake in this area. Techniques described here may be of value in the future study of both the beneficial and deleterious effects of selected medications upon the rate of healing of experimental gastric ulcers. As ulcers in the antrum may respond differently from ulcers in the body of the stomach to exogenous stimulants, the exact location of an experimental ulcer should be noted in every experimental protocol.

SUMMARY

Chronic gastric ulcers were created in the antrum and the body of the dog stomach by a transmural injection of dilute acetic acid, thereby eliminating the need for gastrotomy. These ulcers were studied twice weekly by gastroscopy and gastric photography with the use of a fiberoptic gastroscope with camera, until autopsy was performed at one, three, or six weeks when gross and histologic correlations were made. Normal healing in 13 dogs was contrasted to healing altered by aspirin (eight dogs) or histamine (eight dogs).

This technique of ulcer production with acetic acid and the subsequent study thereof by serial gastroscopy provided an excellent model for studying the effects of selected medications upon ulcer healing. Endoscopy was well tolerated by all dogs and facilitated accurate serial measurements, as compared to autopsy observations made immediately following the last gastroscopic examination. The ulcers of the control group reached maximal size at one week (UI = 154) and were all completely healed at six weeks. Histamine delayed the healing of ulcers in both the body and the antrum of the stomach and produced multiple diffuse erosions which were most severe in the antrum. Aspirin delayed healing of ulcers in the body of the stomach only and produced multiple erosions which were confined to the body. No erosions were seen in the control group. The role of mucin deficiency as judged by decreased PAS stain of the superficial gastric mucosa in the aspirin and histamine groups is discussed.

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Further Observations on Influence of Temperature on Growth of and Toxin Formation by Clostridium botulinum. DR. F. W. TANNER, DR. P. R. BEAMER and DR. C. J. RICKHER (*Urbana, Illinois*).

Certain foodstuffs must be stored under adequate refrigeration to keep them in good condition. It is necessary, therefore, to know what adequate refrigeration is for any particular food-poisoning or spoilage microorganism.

Development of *Clostridium botulinum* in non-acid foods (peas, green beans, asparagus, spinach, sausage and ground beef) and in certain acid foods (cherries, peaches and black raspberries, pH of which was more acid than 4.5) were used. They were inoculated with detoxified spores of 12 strains of *Clostridium botulinum*, four each of types A, B and C. Between

20 and 60 million spores were deposited on each sample of food. The foods were then frozen in a previously cooled, well insulated chamber with solid carbon dioxide at −14°C. (7.2°F.) to −20°C. (4°F.) and held at this temperature for about 3 hours. They were then stored in the constant temperature incubators and examined for presence of toxin after 4 and 14 days.

At 37°C. (98.6°F.) the foods consistently showed presence of toxin. This temperature was used as a control to show that toxin would be formed under favorable conditions. At 20°C. (68°F.) toxin was formed after 4 days in all of the non-acid foods. All the acid foods were negative except cherries which showed presence of toxin after 14 days. At 10°C. (50°F.) there was marked inhibition of toxin formation. It was demonstrable in peas, asparagus and ground beef after 14 days and in spinach after 4 days. Toxin was not formed in 14 days in any of the foods stored at 5°C. (41°F.). These data indicate that if frozen foods are properly handled, they are probably as safe as fresh foods.

Discussion by DR. S. C. PRESCOTT (Cambridge, Massachusetts):

Several years ago we obtained similar results with the same organisms on vegetables. Toxin is not formed in foods kept in the frozen state.

Aust J Sci. Research B4 283-90, (1951)

VOLATILE PRODUCTS OF APPLES

I. IDENTIFICATION OF ACIDS AND ALCOHOLS

By Adrienne R. Thompson*

[*Manuscript received September* 19, 1950]

Summary

The volatile substances given off to the air by Granny Smith apples at 20°C. were condensed at low temperature. The acids obtained on saponification were found to be virtually free from carbonyl, hydroxy, or unsaturated groups. By conversion to hydroxamic acids followed by chromatographic separation on paper they were identified as formic, acetic, propionic, butyric (probably normal), valeric, and caproic acids. All these acids were shown to be present in the esterified form. Formic and acetic acids were shown to occur in both the free and esterified forms. All these acids do not necessarily occur in every sample of volatiles. For instance, formic acid has been found in some samples but not in others.

The alcohols obtained on saponification were found to be predominantly primary and saturated and the major constituents were found to be methanol, ethanol, and *n*-propanol. Ethanol and *n*-propanol were identified by paper chromatography after conversion to hydroxamic acids and methanol by a specific colour test.

I. Introduction

There is evidence that the volatile substances given off to the atmosphere by apples are responsible for superficial scald, a functional disorder of cold-stored fruit (Brooks, Cooley, and Fisher 1919). A systematic study of these volatile substances is being made and this paper describes methods for the identification of the acids and alcohols present in free and esterified forms.

Power and Chesnut (1920) identified methyl, ethyl, and amyl esters of formic, acetic, caproic, and caprylic acids in the steam distillate of the parings of certain apple varieties.

White (1950) found esters of formic, acetic, propionic, butyric, and caproic acids in a concentrate of volatiles distilled from apple juice. Methanol, ethanol, 2-propanol, and butanol were identified as the alcoholic components of the esters. A high proportion of the volatile concentrate was composed of free alcohols which were identified as methanol, ethanol, *n*-propanol, 2-propanol, butanol, *iso*butanol, *d*-2-methyl-1-butanol, and hexyl alcohol. The carbonyl compounds comprised acetaldehyde, acetone, caproaldehyde, and 2-hexenal.

Of the substances given off to the atmosphere by fresh apples at ordinary temperatures, carbon dioxide, ethylene (Gane 1935), and acetaldehyde (Power and Chesnut 1920) have been identified. Walls (1942) has given evidence for the presence of esters of amyl alcohol and of formic and acetic acids among

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the volatile substances from whole Lane's Prince Albert apples after absorption on granular calcium chloride.

In the present work the volatile substances given off to the air by Granny Smith apples at 20°C. were collected by condensation at low temperatures and the constituent acids, free and esterified, and alcohols, free and esterified, were identified.

II. Free and Esterified Acids

(*a*) *Collection and Preparation of Sample*

Air, purified by combustion of organic matter and absorption of carbon dioxide, was passed over 10 kg. of Granny Smith apples at 20°C. The volatiles were condensed for two to five days in a spiral absorber cooled in liquid oxygen. The esters were hydrolysed by addition of 0.2N sodium hydroxide. The alcohols were removed by distillation from the alkaline solution, which was then acidified and the acids distilled. A preliminary test indicated that acids up to decanoic would be recovered in this procedure. The acid distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of all the acids, both free and esterified, were thus obtained.

Tests were first made to determine whether these acids were predominantly simple saturated fatty acids or whether they contained carbonyl, hydroxy, or unsaturated groupings.

(*b*) *Test for Carbonyl Group*

The sensitive method of Friedman and Haugen (1943) for the estimation of carbonyl compounds was used. This method measures the colour produced by reaction with 2,4-dinitrophenylhydrazine in acid solution followed by the addition of sodium hydroxide. The colorimeter readings were converted to equivalents of carbonyl compounds, using pyruvic acid as standard. The mean molecular weight of the mixed sodium salts was assumed to be equal to that of sodium butyrate. This assumption was justified by the identification of the individual acids. The tests indicated not more than one carbonyl group in 1400 moles of mixed acids.

(*c*) *Test for Hydroxyl Group*

The hydroxy content of the mixed sodium salts was estimated by acetylation. The mixed sodium salts (20 mg.) and acetyl chloride (15 drops) were allowed to stand for 15 minutes. Water (approximately 2 ml.) was added and allowed to stand for 30 minutes to decompose the excess acetyl chloride. The acetyl derivatives were separated by extraction with ether. The ether extract was treated with hydroxylamine in alkaline solution to give acethydroxamic acid, which was estimated colorimetrically (Thompson 1950). Lactic acid was used to standardize the method. The test indicated not more than one hydroxy group in 165 moles of mixed acids. The hydroxy content may be appreciably less, as small amounts of anhydrides may resist hydrolysis and be included in the determination.

(*d*) *Test for Unsaturation*

With the small amount of material available, the only satisfactory method of determining unsaturation was found to be hydrogenation with a palladium catalyst in a Warburg flask with manometric estimation of the hydrogen absorbed (Milton and Waters 1949). The figure obtained indicated a ratio of one mole of hydrogen (corresponding to one double bond) to 385 moles of mixed acids.

(e) *Identification as Hydroxamic Acids*

The sample of sodium salts was converted to a mixture of hydroxamic acids in the following manner:

Thionyl chloride (0.2 ml.) was added to the dry sodium salts (10 mg.) and allowed to stand for 15 minutes. Absolute ethyl alcohol (5 ml.) was added and allowed to stand for a further 30 minutes. Water was added and, after allowing several minutes for the decomposition of the thionyl chloride, the esters were extracted from the solution with ether (30 ml.). The ether solution was dried over calcium chloride and neutralized with alcoholic sodium hydroxide. The hydroxamic acids were formed by reaction with hydroxylamine as described in an earlier paper (Thompson 1950). The efficiency of conversion of carboxylic to hydroxamic acid was found to be approximately 70 per cent. with *n*-butyric acid.

The chromatographic separation of the hydroxamic acids was carried out as described previously (Thompson 1951). By comparing the R_F values with those of known hydroxamic acids run on the same paper the acids derived from apples were identified (Plate 1 and Plate 2, Fig. 1). The acids are generally described by the older names which only indicate number of carbon atoms, as in most cases it was impossible to distinguish between isomers.

The relative proportions of the acids varied somewhat with different samples. A sample examined in 1949 was shown to contain acetic, propionic, butyric, valeric, and caproic acids (Plate 1*d*). The chromatogram was obtained with benzene-acetic acid. This test did not prove the absence of formic acid, as in 1949 a solution of ferric perchlorate containing excess perchloric acid was used to develop the chromatograms. Under these conditions formhydroxamic acid is unstable. However, by means of a separate colorimetric test based on reduction to formaldehyde (Grant 1948) and reaction with chromotropic acid (MacFadyen 1945), it was shown that the proportion of formic acid was negligible.

A sample from less mature apples examined in 1950 was found to contain formic and acetic as predominant acids (Plate 2, Fig. 1*b*). A small amount of propionic acid and only traces of higher acids were present. Butanol-acetic acid was used for this test. The spots were developed with ferric chloride to which formhydroxamic acid is quite stable. The chromotropic acid test for formic acid was strongly positive.

III. Esterified Acids

For identification of esterified acids the volatile substances from apples were more conveniently condensed in ether (30 ml.) in a spiral absorber cooled in a mixture of solid carbon dioxide and ethanol. The efficiency of collection of esters in this absorber was about 95 per cent. The ether layer was separated from the water layer and dried. The esters were converted directly to hydroxamic acids by reaction with hydroxylamine (Thompson 1950), and identified by paper chromatography as already described.

For each sample of apples examined all the acids obtained on hydrolysis were represented in the esters. The esterified acids from the 1949 sample of apples were found to include acetic (trace), propionic, butyric (major constituent), valeric (trace), and caproic acids. This is shown in Plate 3, Figure 1 (caprylic alcohol-oxalic acid) and Plate 1*b* (benzene-acetic acid). In Plate 3, Figure 2 (benzene-acetic acid), the absence of higher acids is demonstrated. Storage of these apples at 0°C. for various periods up to 30 weeks made no significant difference to the composition of the esterified acids.

Table 1

R_F values of butyro-hydroxamic acids from esters

Paper	Test Solutions		
	Normal Butyrate	"Apple" Butyrate	*iso*Butyrate
1	0.127	0.125	0.133
	0.148	0.143	0.148
	0.143	0.142	0.160
	0.134	0.140	0.168
2	0.136	0.152	0.162
	0.150	0.151	0.148
	0.137	0.134	0.145
	0.104	0.117	0.136
3	0.112	0.120	0.135
	0.122	0.132	0.132
	0.127	0.125	0.158
	0.125	0.125	0.147

From the less mature apples examined in 1950 only formic acid and acetic acid were identified amongst the esterified acids. Butanol-acetic acid was used as solvent mixture (Plate 2, Fig. 1*a*).

An attempt was made to determine whether the butyrate present was normal or *iso*butyrate. It was possible, by paper chromatography with benzene and acetic acid, to effect a small separation of normal and *iso*butyrohydroxamic acids. By statistical treatment of the R_F values (Table 1) of the two isomers and of the butyrohydroxamic acid derived from apples, it was

shown that the probability of the "apple" butyrate being *iso*butyrate is less than 0.001, whereas the probability of it being normal butyrate is greater than 0.2 (Table 2).

TABLE 2

ANALYSIS OF VARIANCE OF DATA IN TABLE 1

Source of Variation		D.F.	Mean Squares	Ratio
Between solutions (*n*-butyrate, "apple" butyrate and *iso*-butyrate)	(S)	2	0.000950	16.10***
Between papers	(P)	2	0.000550	9.32**
S × P		4	0.000024	N.S.
Between replicates within papers	(R)	9	0.000337	5.71***
R × S		18	0.000059	
Total		35		
"Apple" butyrate v. *iso*butyrate		1	0.001080	18.31***
n-Butyrate v. (mean of "apple" and *iso*butyrate)		1	0.000820	13.90**
"Apple" butyrate v. *n*-butyrate		1	0.000070	1.19 (N.S.)
*iso*Butyrate v. (mean of "apple" butyrate and *n*-butyrate)		1	0.001830	31.02***

N.S. = not significant; * = significant at 5 per cent. level; ** = significant at 1 per cent. level; *** = significant at 0.1 per cent. level.

IV. Free Acids

The volatile substances from apples were collected in ether cooled in a solid carbon dioxide-alcohol bath. The ether solution, with a similar volume of water, was neutralized with 0.1N sodium hydroxide using phenolphthalein as indicator and shaking vigorously throughout. The aqueous layer was separated and evaporated to dryness. The sodium salts of the free apple acids were converted to hydroxamic acids as described in Section II and separated by paper chromatography. Only the free acids from the less mature apples in 1950 were examined by this procedure. Using phenol or butanol-acetic acid (Plate 2, Fig. 2), formic and acetic acids were identified but no higher acids. Formic acid was also identified by the chromotropic acid test.

It had previously been shown that the esterified acids from these apples were mainly formic and acetic. It appears, therefore, that formic and acetic acids, when present, occur free as well as esterified. The higher acids predominated in the esterified acids from the more mature apples in 1949, but unfortunately no attempt was made to identify the free acids from these apples. Hence there is no evidence that the higher acids occur free but the possibility is not excluded.

V. Free and Esterified Alcohols

(*a*) *Preparation of Solution*

The distillate from the alkaline solution of saponified volatiles collected in 1949 (see Section II (*a*)) was used for the identification of alcohols. A micro-hydrogenation test indicated negligible unsaturation, amounting to approximately one double bond in 600 moles. This calculation was based on the weight of sodium salts obtained on oxidation, assuming an average molecular weight equal to sodium acetate.

A colorimetric test (Friedman and Haugen 1943) showed the presence of trace amounts of carbonyl compounds. The distillate was therefore treated with the 2,4-dinitrophenylhydrazine solution and distilled under reduced pressure, with ice water in the condenser, to obtain a distillate free from carbonyl compounds.

(*b*) *Test for Secondary Alcohols*

An aliquot of the carbonyl-free distillate was oxidized with dilute chromic acid and again distilled. The distillate of oxidized products was free from carbonyl compounds, indicating the absence of secondary alcohols before oxidation.

(*c*) *Identification as Hydroxamic Acids*

The carbonyl-free solution of primary alcohols was oxidized with chromic acid and the acids distilled off. The distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of the fatty acids were converted by hydroxamic acids by the method previously described (Section II (*e*)).

Hydroxamic acids containing two, three, and six carbon atoms (the last only in traces) were identified on the chromatogram (Plate 1*c*). The presence of ethanol and *n*-propanol and a trace of six-carbon alcohol in the original solution was thus established. The use of ferric perchlorate-perchloric acid solution as developer prevented the detection of formhydroxamic acid and hence methanol in the solution of alcohols.

However, the presence of methanol was shown by a specific test on a solution of apple alcohols. After oxidation with permanganate (Feigl 1943) the solution gave a positive test for formaldehyde with chromotropic acid. No formaldehyde was detected before oxidation.

VI. Discussion

The author has found saturated fatty acids containing one, two, three, four, five, and six carbon atoms and saturated alcohols containing one, two, three, and six carbon atoms in the volatiles from fresh apples. It is probable that each acid and alcohol can occur both in the free and esterified form. All the acids have been found as esters, and formic and acetic acids have also been found free. The higher acids—free and esterified—were virtually absent from the only sample tested for free acids.

No other acids were found by White (1950) and Walls (1942), but an acid with eight carbon atoms was found by Power and Chesnut (1920). Walls (1942) and Power and Chesnut (1920) found amyl alcohol, and White (1950) found alcohols containing one, two, three, four, five, and six carbon atoms. Although White's work was based on the volatile fraction of apple juice, it is probable that every member of this series was present in the original fresh apples from which the juice was derived, as all except the four-carbon alcohols have been found either by Walls (1942) or the author in the volatiles from whole fresh apples.

The volatile fraction of apple juice analysed by White (1950) was found to contain 92 per cent. of free alcohols, 6 per cent. of carbonyl compounds, and only 2 per cent. of esters. The ratio of esters to free alcohols in the volatiles evolved by fresh apples would probably be higher since the esters may more readily pass through the lipoid phase of the cuticle and be preferentially evolved. Moreover, in preparation of the juice, part of the esters may be retained in the pressed residue, and there may also be subsequent loss of esters by hydrolysis.

The author's results, in conjunction with those of other workers, provide definite evidence that saturated acids and alcohols containing one, two, three, four, five, and six carbon atoms can occur in the volatiles from fresh apples and enter into a large variety of ester combinations. The relative proportions of the different components may vary from sample to sample, thus reflecting the differences in aroma and flavour associated with variety, maturity, and other factors.

The presence of homologous series of acids and alcohols with both odd and even numbers of carbon atoms is unusual among natural products. Products containing only even-numbered acids are more common, but recently Weitkamp, Smiljanic, and Rothman (1947) have demonstrated the presence of a complete series of odd-numbered fatty acids with seven to 17 carbon atoms, as well as even-numbered acids, in human hair fat.

Some comments may be made on the mechanism by which these substances are formed in the apple. Presumably each alcohol is interconvertible with the corresponding acid by oxidation and reduction. The current biochemical theories for the synthesis of fatty acids *in vivo* account only for the production of even-numbered straight chains. An important contribution to the mechanism of fatty acid synthesis has been made by the recent discovery of Stadtman and Barker (1949) that cell-free extracts of *Clostridium kluyveri* catalyse the linkage of ethanol and acetate to fatty acids of four and six carbon atoms. Both ethanol and acetate are closely related to the respiratory cycle. The linkage of odd-numbered acids or alcohols with two-carbon fragments has not been investigated but there remains the possibility of obtaining the higher odd-numbered acids by this means. A one-carbon fragment may be necessary for the initial condensation and this could be readily provided as methanol, which is always present in apple tissue as the methyl ester of pectic acid. The reduction of three-carbon intermediates of respiration to propionic acid is also possible. To obtain branched chains, linkage of fragments at other than end

groups would be necessary. This mechanism could not account for the formation of the secondary alcohol 2-propanol, which was found by White (1950) in the volatile concentrate from apple juice.

VII. Acknowledgments

The author wishes to thank Dr. F. E. Huelin for his interest and advice in this work, Mr. G. Ferris for the statistical analyses, and Mr. Bruce Kennett for technical assistance. Thanks are also due to Dr. A. L. G. Rees, Mr. G. R. Hercus, and Dr. J. D. Morrison for undertaking mass spectrometric analyses of samples of apple volatiles. Their results are described in Appendix I.

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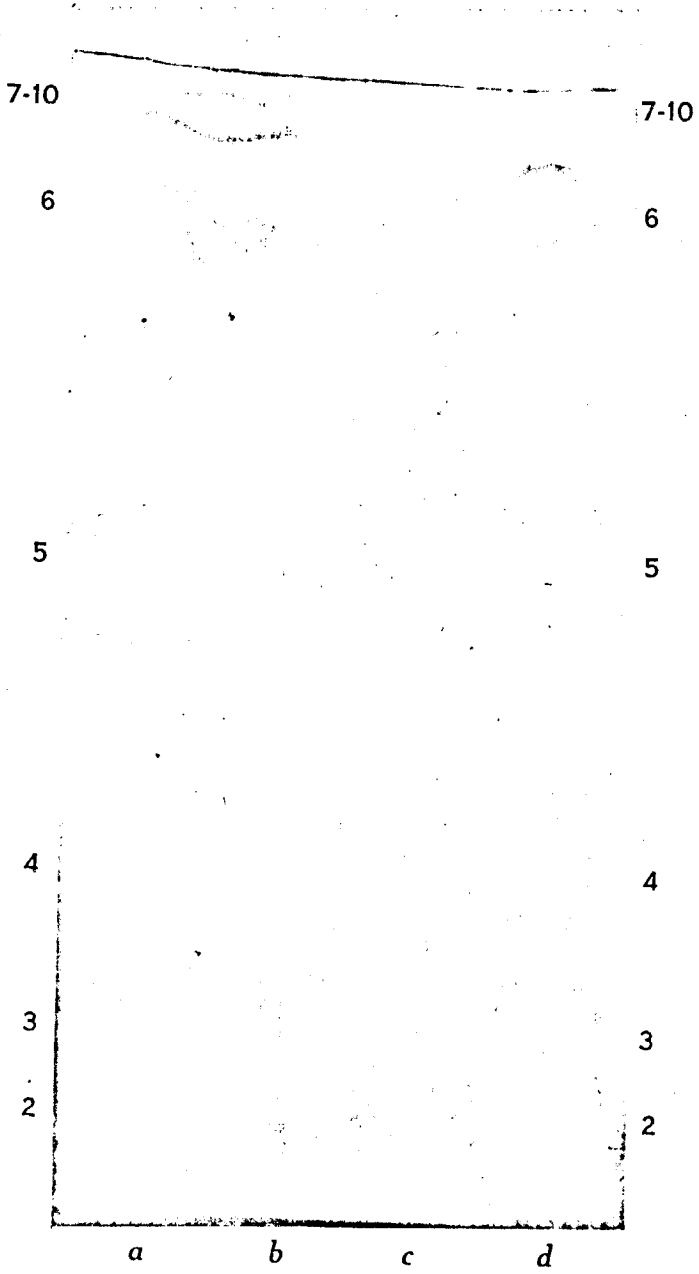
Appendix I

Mass Spectrometric Analyses

By G. R. Hercus* and J. D. Morrison*

In conjunction with the work reported in the above paper, a mass spectrometric examination of the apple volatile products was made, giving results in substantial agreement with those obtained above.

* Chemical Physics Section, Division of Industrial Chemistry, C.S.I.R.O., Melbourne.



Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

(*a*) An artificial mixture of esters containing acids with one to ten carbon atoms;
(*b*) Esterified acids from apples (1949);
(*c*) Free and esterified alcohols from apples (1949);
(*d*) Free and esterified acids from apples (1949). The appearance of two

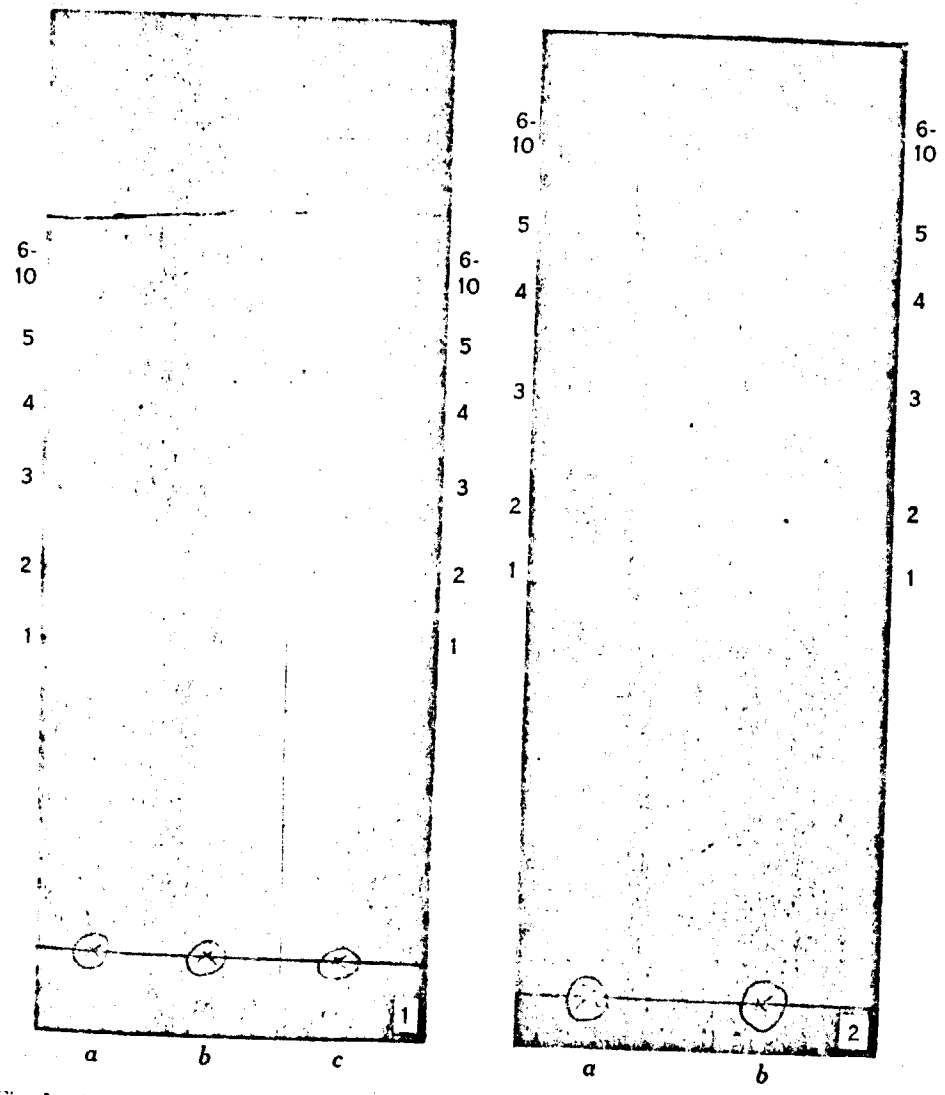


Fig. 1.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

(*a*) Esterified acids from apples (1950);
(*b*) Free and esterified acids from apples (1950);
(*c*) An artificial mixture of esters containing acids with one to ten carbon atoms.

Fig. 2.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

(*a*) Free acids from apples (1950);
(*b*) An artificial mixture of esters containing acids with one to ten carbon atoms.

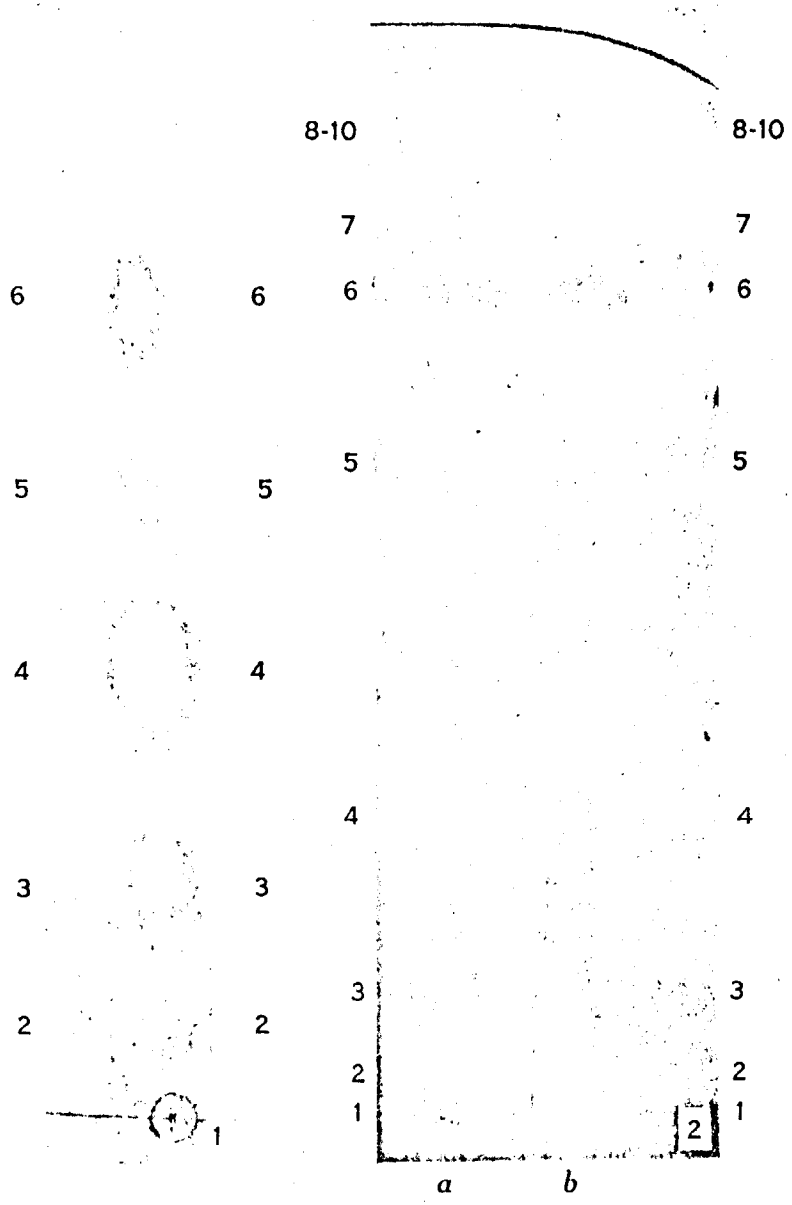


Fig. 1.—Paper chromatogram run with caprylic alcohol-oxalic acid, showing ferric hydroxamate spots derived from esterified acids from apples (1949).

Fig. 2.—Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

(*a*) Esterified acids from apples (1949);

(*b*) An artificial mixture of esters containing acids with one to ten carbon atoms.

The apple volatile products were collected as two samples, the less volatile (1) in an ice-calcium chloride trap, and the more volatile (2) in a liquid-air trap.

The mass spectra of these samples were measured, when it was found that the first was heavily contaminated with water, and the second with CO_2. It did not prove possible to separate the water by fractionation, but the CO_2 could be removed by this method.

The mass spectrum of the more volatile sample (2) after fractionation was complex, prominent peaks being observed at masses 74, 102, 116, 130, 136, 206. It was thought that the samples contained aliphatic esters, and examination of the spectra of a number of such esters with parent peaks at these values indicated that ethyl *n*- and *iso*butyrate, and ethyl *n*- and *iso*valerate were more probable than esters such as *n*-butyl acetate. This evidence was, however, not conclusive.

TABLE 3

COMPARISON OF MASS SPECTRA OF SAMPLES *A* AND *B* WITH THOSE OF ALIPHATIC MONOCARBOXYLIC ACIDS

Mass	Ion Peak Heights					
	Sample	Acetic	Propionic	*n*-Butyric	*n*-Valeric	Sum of Mass Spectra of Pure Acids
	Sample *A*					
43	249	49	110	265	27	451
45	357	45	180	133	14	372
60	466	29		375	47	451
73	205		120	106	16	242
74	205		198	6	4	208
87	20			6	1	7
88	17			16		16
	Sample *B*					
43	674	692	37			729
45	588	625	61			686
60	422	422				422
73	43		40			40
74	66		66			66

At this stage the volatiles of type RCOOR′ were hydrolysed to the corresponding alcohols R′OH and acids RCOOH, which were separated by distillation. The acids were converted to the sodium salts, sample *A*, and the alcohols oxidized with chromic acid to the corresponding acids, these also being converted to the sodium salts, sample *B*.

The free acids were obtained when desired for analysis by treatment of the Na salts in vacuo with vacuum-dried H_3PO_4, and the mass spectra of the acids in samples *A* and *B* measured.

The mass spectra of purified samples of the aliphatic acids acetic, propionic, *n*- and *iso*butyric and *n*- and *iso*valeric were obtained and compared with the spectra of samples *A* and *B*. (Table 3.) The *iso*- acids gave large peaks at mass numbers where there were none observed in the spectra of the acids from samples *A* and *B*, so it was assumed that only normal acids were present in these.

The agreement obtained between the sum of the patterns of the aliphatic acids and the patterns produced by the samples is not very good; this is probably due to the fact that the acids employed for obtaining the calibration spectra were not of high purity. Nevertheless, the results indicate that sample *A* probably contains acetic, propionic, *n*-butyric, and *n*-valeric acids, and that sample *B* contains acetic and propionic acids as major constituents.

The esters present in the original sample would therefore be ethyl and propyl acetate, propionate, *n*-butyrate, and *n*-valerate.

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RELATIONSHIP OF RUMEN VOLATILE ACIDS, BLOOD GLUCOSE AND PLASMA NONESTERIFIED FATTY ACIDS IN SHEEP[1]

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THE metabolic importance of plasma nonesterified fatty acids (NEFA) as a source of available energy for oxidative metabolism was first suggested by Gordon and Cherkes (1956) and Fredrickson *et al.* (1958). These workers observed that the circulating concentration of NEFA in humans was related to the nutritional state of the subject and increased markedly during fasting. Increased glucose utilization by adipose tissue has been observed to decrease the release of NEFA and increase the rate of lipid synthesis (Bally *et al.*, 1960). Since ruminants derive a considerable portion of their metabolizable energy from volatile fatty acids produced in the rumen and normally have blood sugar concentrations about 50% of that in nonruminants, a study of plasma NEFA levels in sheep was of interest. Plasma NEFA levels in ruminants have been studied by Annison (1960) and Patterson (1963), who observed that circulating levels increase with fasting and decrease following infusions of glucose or propionate.

The object of the present experiment was to study the relationship between plasma NEFA, blood glucose and rumen volatile fatty acids in fed and fasting sheep.

Methods and Materials

Experimental Animals. Wether lambs weighing between 36 and 41 kg. were accustomed to handling and blood sampling before they were used in the experiments. The daily ration consisted of 907 gm. of the following mixture: ground corn, 35%; ground corn cobs, 12%; ground alfalfa hay, 42%; soybean meal, 5%; molasses, 5%; salt, 0.5%; and mineral premix, 0.5%. The animals were housed indoors in individual pens, had free access to water and were fed two times per day.

Rumen samples were obtained by means of a stomach tube and the suction strainer described by Raun and Burroughs (1962). One milliliter of 5% mercuric chloride (w/v) was added immediately to 20 ml. of rumen fluid to inhibit fermentation. Blood samples were taken from the jugular vein. Heparin was used as the anticoagulant.

Chemical Analysis. The concentration of rumen volatile acids was determined by steam distillation and titration with standardized 0.05 N potassium hydroxide (Raun and Burroughs, 1962). The concentration of glucose in whole blood was determined by procedures described by Somogyi (1952) using the arsenomolybdate reagent (Nelson, 1944). Plasma NEFA levels were estimated by a modification of the procedure of Dole (1956). Hydrochloric acid (1 N) was substituted for sulfuric acid in the extration mixture originally described. After the heptane extracts had been dried under vacuum at 65° C. the residue was dissolved in 95% ethanol, and the acids were titrated to the green color of thymol blue with a standardized 0.015 N sodium hydroxide solution.

Effect of Fasting. Samples of blood and rumen fluid were obtained from four lambs immediately prior to feeding (14 hr. after previous feeding) and at 2, 4, 8, 24 and 48 hr. after feeding. Patterson (1963) observed that the stress of handling and multiple blood sampling affected the levels of plasma NEFA in sheep. To investigate whether stress of handling was responsible for the small decrease in levels of NEFA observed 4 hr. after the lambs had been fed (figure 1), two lambs were bled once before feeding and again 4 hr. after feeding, while two additional lambs were bled only once, at 4 hr. after feeding. The following week each pair of animals was subjected to the other bleeding regimen and the experiment was repeated.

Effect of Injecting Rumen Volatile Acids. Three lambs were used in this series of experiments. A cross-over design was used, so each animal received each of the experimental treatments. In the first series of experiments the treatments were 50 ml. of 0.85% sodium chloride (w/v), 1.2 molar glucose or

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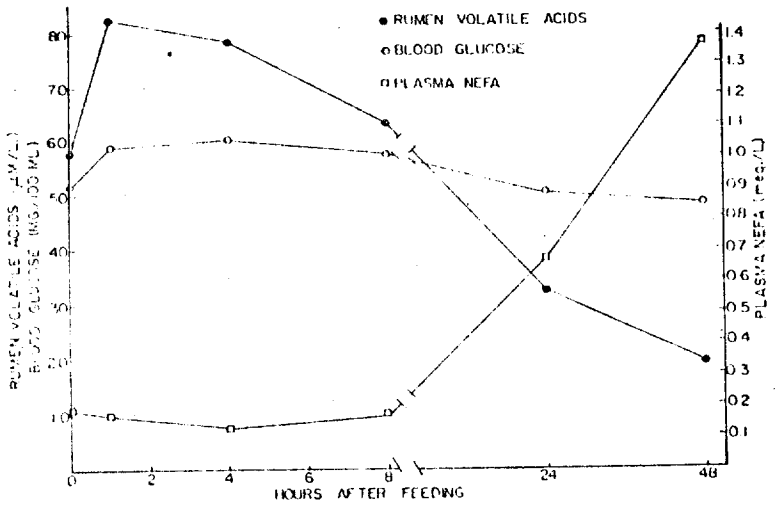

Figure 1. Changes in ruminal volatile acids, plasma NEFA and blood glucose in sheep following feeding.

2.4 molar sodium propionate. In the second series of experiments the treatments were 50 ml. of 0.85% sodium chloride, 2.4 molar sodium acetate or 2.4 molar sodium butyrate. The procedure was to fast the animal for 48 hr., take a blood sample, inject the experimental solution into the jugular vein and take blood samples 1 and 2 hr. following the injection. At least a 2-week period elapsed before the animals were subjected to a different treatment.

Effect of Injecting Insulin. Three lambs were given intravenously 0.1 unit of protamine zinc insulin per kg. of body weight 48 hr. following feeding. Three similar lambs which had been given 0.85% sodium chloride (w/v) served as controls.

Results

Effect of Fasting. During the course of these studies it was possible to measure levels of plasma NEFA in several animals at various times after feeding. Concentrations of plasma NEFA (meq. l. in 36- to 41-kg. wether lambs at 4, 24, and 48 hr. following feeding averaged 0.11 (18 observations), 0.60 (10 observations) and 1.32 (45 observations), respectively.

The relationship between concentration of volatile acids in the rumen, blood glucose and plasma NEFA is shown in figure 1. The concentration of rumen acids increased ($P<.01$) to a peak of 83.3 mmoles l. 1 hr. after feeding, remained relatively constant for 4 hr. and then declined ($P<.01$) to 19.3 mmoles l. 48 hr. after feeding. The concentration of plasma NEFA tended to decrease after feeding and then increase significantly ($P<.01$) at 24 and 48 hr. The increase in plasma NEFA coincided with the decrease in rumen acid concentration. No significant differences were observed in concentration of blood glucose following feeding.

In the second experiment the concentration of NEFA before feeding was 0.18 meq./l. Four hours after feeding the concentration of plasma NEFA was the same (0.11 meq. l.) in the lambs which had been bled before feeding and in those which had not been bled prior to feeding. In each experiment the level of plasma NEFA tended to be lower 4 hr. postprandial, but the means were not significantly different. Analysis of pooled data from the two experiments (eight lambs per group) indicated that levels of plasma NEFA were lower ($P<.05$) 4 hr. after feeding, as compared with levels found before feeding. The results of the second experiment suggest that the decline in level of plasma NEFA observed following feeding was not brought about by handling or excitement of the animals.

Effect of Injecting Rumen Volatile Acids. The changes in plasma NEFA and blood glucose in sheep fasted for 48 hr. followed by injection of individual rumen volatile acids or glucose are shown in figure 2. One and 2 hr. after injection of glucose the concentra-

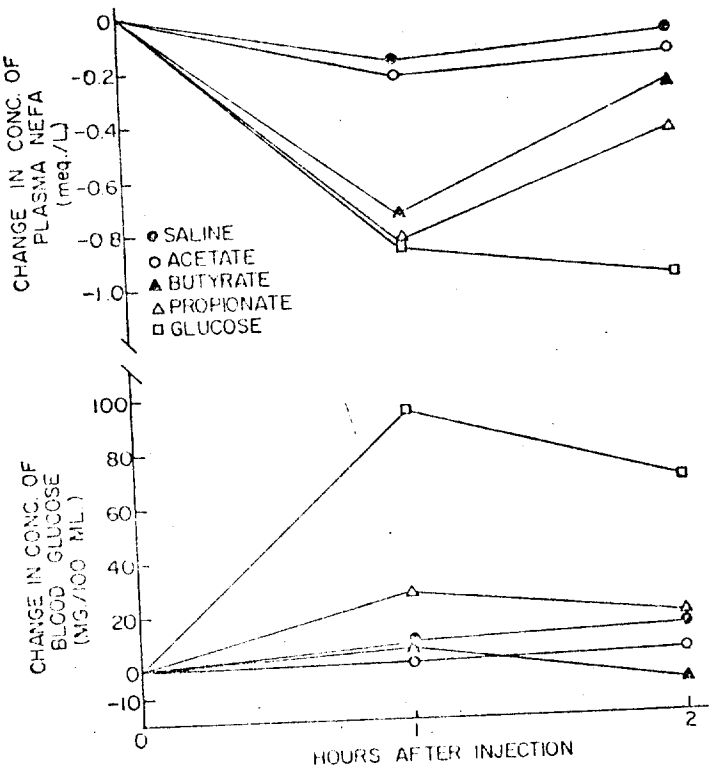

Figure 2. Effect of intravenous administration of glucose (0.06 moles), sodium acetate (0.12 moles), sodium propionate (0.12 moles) and sodium butyrate (0.12 moles) on blood glucose and plasma NEFA in fasting sheep.

tion of blood glucose was increased ($P<.01$) and the level of NEFA was lower ($P<.01$), as compared with the effect of injecting saline. Acetic acid had no significant effect on plasma NEFA or blood glucose during the 2-hr. period following injection. Propionic acid produced an increase in blood glucose ($P<.05$) and a decrease in plasma NEFA ($P<.01$) at 1 hr. following injection. Two hours after injection of propionic acid, plasma NEFA had returned nearly to the level observed prior to injection but still remained significantly ($P<.05$) lower than for that of the saline controls. At 1 hr. following injection butyric acid had not altered the concentration of blood glucose but had depressed ($P<.05$) plasma NEFA. Two hours after butyric acid was injected the concentration of blood glucose was lower ($P<.05$) and the levels of NEFA were similar to those found in lambs injected with saline.

Effect of Injecting Insulin. Following intravenous administration of 0.1 unit of insulin kg. of body wt., the concentration of blood glucose declined from 52 to 34 mg. 100 ml. within 2 hr. (figure 3). The levels of plasma NEFA were depressed 1 hr. following administration of insulin, but then tended to increase ($P<.05$) to levels higher than the fasting levels observed prior to administration of insulin. The same quantity of insulin had no effect on blood glucose or plasma NEFA in fed sheep.

Discussion

The changes in concentration of plasma NEFA and blood glucose observed in the present study are in agreement with results with sheep by Annison (1960) and with ewes, calves and cows by Patterson (1963). The slow decline in blood glucose during the fasting of sheep suggests that the concentration of blood glucose is not *in toto* responsible for the observed rise in levels of plasma NEFA. It appears that the increased concentration

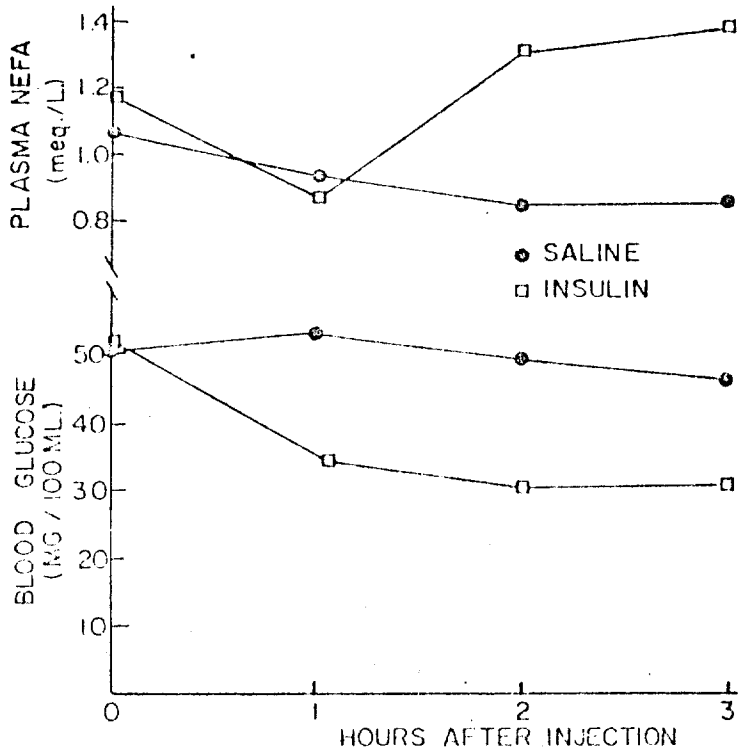

Figure 3. Effect of intravenously-injected insulin (0.1 unit/kg. body wt.) on blood glucose and plasma NEFA in fasting sheep.

of plasma NEFA in fasting sheep might be a metabolic response to a decrease in rate of utilization of glucose at the tissue level. Bergman (1963) and Annison and White (1961) have observed that the rate of utilization of glucose is less in sheep fasted for 24 hr. than in fed sheep. Absorption of glucogenic materials from the digestive tract or direct injection of glucose or glucogenic materials such as propionate tends to restore utilization of glucose to normal and consequently to decrease mobilization of depot lipids. Data in the present study support this contention.

Acetic and butyric acids are not considered to result in net synthesis of glucose (Leng and Annison, 1963). However, in the present study butyric but not acetic acid decreased mobilization of body lipids in fasting sheep. The lower level of blood glucose observed 2 hr. after injection of butyric acid suggests that the rate of utilization of glucose may have been increased either directly or indirectly by administration of sodium butyrate.

The initial response to insulin appears to be one of increased glucose utilization, which resulted in decreased blood glucose concentration and a decreased level of plasma NEFA. After available glucose supplies were exhausted following stimulation by insulin, the concentration of plasma NEFA increased.

Experiments conducted by Bergman (1963) and Annison and White (1961) have indicated the central importance of glucose metabolism in sheep, even though little glucose is absorbed from the digestive tract. The present study further suggests the importance of active glucose metabolism for lipogenesis in sheep.

Summary

Concentrations of plasma nonesterified fatty acids in sheep were 0.11, 0.60 and 1.32 meq. l. 4, 24 and 48 hr. following feeding. No significant changes in concentration of blood glucose were observed during 48 hr. after feeding. The increase after feeding in concentration of plasma nonesterified fatty

acids coincided with a decrease in concentrations of volatile acids in the rumen. Intravenous injection of glucose, sodium propionate or sodium butyrate into fasting sheep decreased the level of plasma nonesterified fatty acids within 1 hr. No significant change occurred after injection of sodium acetate. Concentration of blood glucose increased following injection of propionate or glucose, remained unchanged following acetate injection and decreased following butyrate injection.

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GAS CHROMATOGRAPHIC SEPARATION AND DETECTION OF C_1 TO C_3 MONOCARBOXYLIC ACIDS AS THE *p*-SUBSTITUTED BENZYL ESTERS

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SUMMARY

A rapid, sensitive gas chromatographic method for the separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids is presented. The procedure involves reaction of microliter volumes of an ethanolic carboxylic acid solution mixture, down to the 0.1 $\mu g/\mu l$ concentration level, with an appropriate *p*-substituted benzyl halide in a sealed capillary melting point tube at 110°. The esters formed in the reaction mixture are then isothermally eluted from a general-purpose OV-17 glass column.

INTRODUCTION

Separation and/or detection of the lower monocarboxylic acids in foods and in chemical, biological and ecological media has been achieved using spot tests, paper and thin-layer chromatography[1-5], column partition chromatography[6,7] and by gas chromatography as the free acids or after derivatization under a variety of experimental conditions[8-11].

Color tests are often subject to interference and are therefore of limited utility. Column, paper and thin-layer chromatographic techniques are virtually indispensable to the chemist but the lower volatile fatty acids sometimes demand assiduous sample preparation and control of conditions, and may require relatively long development times for optimum separation. Though excellent results are reported in the literature for most of the gas chromatographic methods for the lower carboxylic acids, many require specialized columns which are not always convenient for those control laboratories which routinely process a wide diversity of chemical substances.

Therefore, in this paper we describe a sensitive method for the rapid separation and detection of microgram and submicrogram quantities of formic, acetic and propionic acids by isothermal gas chromatography on an all-purpose 5% OV-17 on Gas-Chrom Q column as their *p*-methylbenzyl, *p*-bromobenzyl or *p*-nitrobenzyl esters.

EXPERIMENTAL

Materials

The carboxylic acids used were of reagent grade quality.

The following *p*-substituted benzyl halides were used: *p*-nitrobenzyl iodide, K & K (95–99%); *p*-bromobenzyl bromide, K & K (95–99%); *p*-methylbenzyl bromide, Eastman (highest purity); *p*-nitrobenzyl bromide, Eastman (highest purity).

p-Nitrobenzyl, *p*-methylbenzyl and *p*-bromobenzyl esters of formic, acetic and propionic acids were obtained commercially where available (*p*-nitrobenzyl acetate, K & K, 95–99%; *p*-methylbenzyl acetate, K & K, 95–99%) or synthesized according to the procedures described below.

Synthesis of p-substituted benzyl propionate and acetate reference materials. Propionic acid or acetic acid (3 g) dissolved in water (5 ml) was neutralized to a phenolphthalein end-point with 20% aqueous sodium hydroxide solution. A few drops of dilute hydrochloric acid were added so that the final solution was faintly acid to litmus paper. To this was added the appropriate *p*-substituted benzyl bromide (1 g) dissolved in alcohol (25 ml) and the mixture was heated under reflux for 3 h. The mixture was evaporated to half volume and then diluted with water. After extraction into ether, the organic layer was dried over anhydrous sodium sulfate, the solvent removed and the residual oil distilled *in vacuo*.

Synthesis of p-substituted benzyl formate reference materials. The *p*-substituted benzyl alcohol (1 g) was heated under reflux in formic acid (20 ml) containing concentrated HCl (three drops) for 2 h. The mixture was cooled, diluted with ether and shaken with several portions of 3% sodium bicarbonate solution. The organic layer was dried over anhydrous sodium sulfate, the ether removed under a flow of dry nitrogen, leaving an oil which on vacuum distillation yielded the desired product. The halide may also be used, but the yields appeared to be considerably lower.

p-Bromobenzyl formate was synthesized according to the above procedure using *p*-bromobenzyl bromide.

Reference solutions

All reference esters were made up to a concentration of 1 $\mu g/\mu l$ in ethanol.

Reagent solutions

The reagent solutions were prepared as follows: Individual carboxylic acid solutions were made up to a concentration of 1 $\mu g/\mu l$ in ethanol. An ethanolic mixture of all three carboxylic acids was made up so as to contain 1 $\mu g/\mu l$ of each component. Solutions of potassium hydroxide, *p*-nitrobenzyl iodide, *p*-methylbenzyl bromide, and *p*-bromobenzyl bromide each contained 3 μg per μl ethanol.

Micro sample preparation and gas chromatography

The carboxylic acid solution (10 μl) was transferred by means of a Hamilton microsyringe into a capillary melting point tube along with potassium hydroxide in ethanol solution (3 μl) and the appropriate *p*-substituted benzyl halide solution (5 μl) described above (in the case of solution 2, 15 μl were used). The tube was flame sealed and incubated at 110° for 1 h.

After cooling, 2 μl of the reaction mixture were injected into a gas chromato-

graph* equipped with a flame ionization detector unit and fitted with a 5% OV-17 on Gas-Chrom Q (100–120 mesh) glass column (4 ft. × $\frac{1}{8}$ in. I.D.) preconditioned at 280° for 24 h. Column and injection port temperatures were, respectively: for the *p*-methylbenzyl series 100° and 225°, for the *p*-bromobenzyl series 120° and 240°, and for the *p*-nitrobenzyl series 145° and 265°. Nitrogen flow was 80 ml/min, hydrogen flow 50 ml/min, column inlet pressure 8 p.s.i.g., and attenuation × 100.

RESULTS AND DISCUSSION

In order to have an adequate method for the identification of lower molecular weight carboxylic acids by gas chromatography, it was desirable to have at least three different derivatives of each acid. The *p*-substituted benzyl esters offered the most advantages as they are readily prepared and are amenable to gas chromatography.

Reference material preparation

When preparing the reference materials, not all of the ester derivatives were commercially available, hence the appropriate ones were synthesized as described. The feasibility of the synthesis routes was established by gas chromatographic comparison of the pure (95–99%) commercial ester—where available—with the vacuum distillate of the same ester prepared by laboratory synthesis. In addition, IR spectral traces were used to confirm the ester linkage by the presence of the carbonyl stretching frequency band near 1740 cm^{-1}. The correct structure for each of the remaining synthesized esters was then inferred *a posteriori* from these data. The gas chromatograms of the esters synthesized for this investigation did indicate that small quantities of impurities (*e.g.* the corresponding alcohol) were present after a single vacuum distillation but further purification did not appear to be warranted.

Micro sample preparation

In the simulated microanalysis of each carboxylic acid with each *p*-substituted benzyl halide, injection of the reaction mixture was subsequently followed by injection of the ethanolic solution of the reference ester and then finally by injection of a 1:1 mixture of the first two (see Fig. 1, chromatograms a, b and c). In every case, gas chromatography of the reaction mixture afforded a peak of identical retention time to that of the reference ester thus confirming the formation of the expected ester in the capillary tube. In a given series, ester retention times increased with molecular weight of the carboxylic acid such that a mixture of the three acids could be easily resolved after the derivatization step. Fig. 1, chromatogram d, clearly demonstrates a typical separation of a formic, acetic and propionic acid mixture after benzylation, in this instance effected with *p*-bromobenzyl bromide and ethanolic KOH at 110°. Table I gives the retention times, calculated from the point of injection, for each ester and for other relevant compounds in a series. The reaction mixtures gave, in addition to a symmetrical ester peak, a sharp secondary "reaction artifact" peak presumably resulting from isomerization or decomposition of the thermally unstable *p*-substituted benzyl halides but the nature of the product was not investigated. Ethanolic solutions of the halides can, however, be stored at 0° for a week without undergoing appreciable

* Research Specialities Co. "600 series". The FID unit was Model 660.

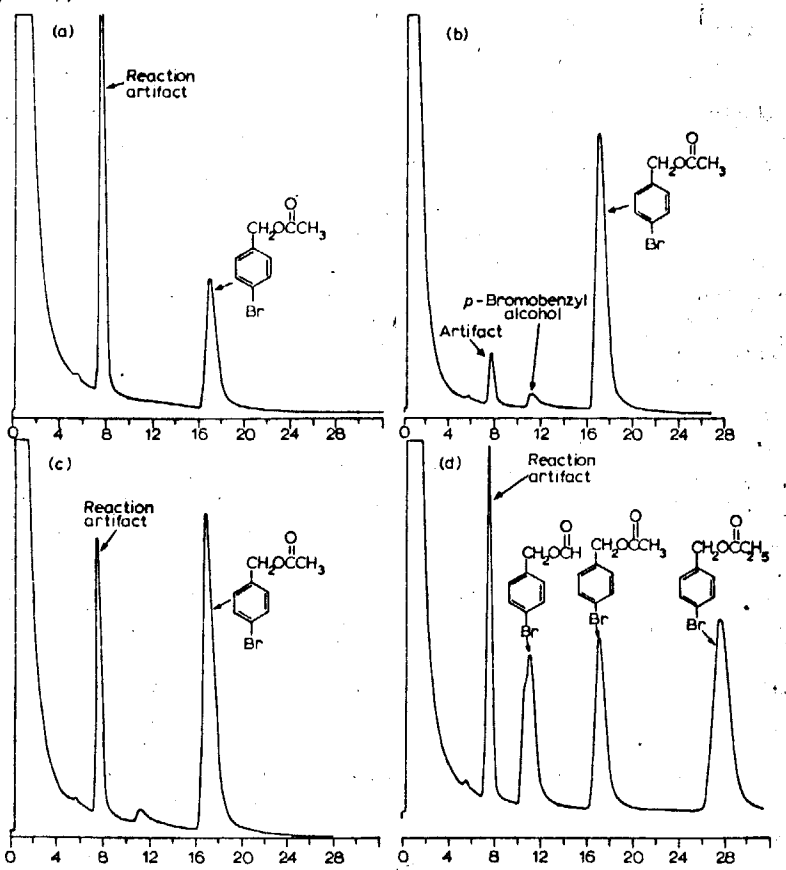

Fig. 1. (a) Reaction mixture (micro preparation using acetic acid solution). (b) Reference standard solution. (c) Reaction mixture of (a) + reference standard of (b) 1:1. (d) Reaction mixture (micro preparation using three-component acid solution).

TABLE I

RETENTION TIMES (min) OF *p*-SUBSTITUTED BENZYL FORMATE, ACETATE AND PROPIONATE ESTERS AND OTHER RELEVANT RELATED COMPOUNDS

	p-Methylbenzyl (column temp. 100°)	*p-Bromobenzyl (column temp. 120°)*	*p-Nitrobenzyl (column temp. 145°)*
Formate	8.4	10.9	11.0
Acetate	15.1	17.0	15.3
Propionate	26.3	27.4	22.8
Alcohol	6.6	10.2	12.6
Halide	9.3	12.7	25.5
Artifact	5.7	7.5	7.2

degradation. The presence of some unreacted *p*-nitrobenzyl iodide and its hydrolysis product *p*-nitrobenzyl alcohol was noted in the reaction mixtures of the *p*-nitrobenzyl esters. The iodide was found to be superior to the bromide in this series giving a cleaner reaction mixture and generally enhancing ester formation. In each series, the equilibrium constant for the esterification reaction appeared to be smallest for formic acid, this being suggested by the relative peak heights of the formate, acetate and propionate esters. Optimum results were realised with *p*-bromobenzyl derivatization; peaks were sharp and symmetrical and, aside from the "reaction artifact", the formation of the ester in the capillary tube was attented by no additional observable side reactions except in the case of the formate where, under the stated conditions, a small shoulder peak suggested the presence of some of the corresponding alcohol (see Fig. 1, chromatogram d).

With the appropriate attenuation setting, as little as 10 ng of reference ester were detected. However, in the micro preparation, detection of the carboxylic acid was not usually successful with sample solution concentrations lower than 0.1 $\mu g/\mu l$. The presence of appreciable amounts of water in the reaction mixture promoted virtually complete hydrolysis of the halide to the benzyl alcohol so that aqueous carboxylic acid solutions at the 1 $\mu g/\mu l$ level afforded only a very small ester peak. Nevertheless, detection of the acid was still possible when aqueous solutions of this concentration were diluted tenfold with ethanol and treated as described.

Though a number of chromatographic methods have already been proposed for the detection and separation of C_1 to C_3 monocarboxylic acids in various media such as in foods, in cigarette smoke or as pollutants in river waters, the present method might prove useful for such determinations carried out by control laboratories which, of necessity, employ general-purpose columns in their day to day operation.

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THE ACUTE TOXICITY OF COMMERCIAL PENICILLIN

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THE large volume of clinical data collected during the relatively short time that penicillin has been used presents extremely strong evidence of the innocuous nature of this drug. The clinical evidence has been obtained on a variety of diseases through the efforts of the Office of Scientific Research and Development in carefully organized clinical studies. Such an accomplishment in normal times would have required years of effort by individual investigators. From the original studies of Chain, et al.[1] and Abraham, et al.,[2] it has been apparent that even with relatively crude preparations there was little to indicate any contraindication to its use in man. Later studies by Hobby, et al.,[3] Herrell, et al.,[4, 5] Blake and Craige,[6] and Rammelkamp and Keefer,[7] have all substantially confirmed the fact that at least so far as penicillin sodium is concerned, little, if any, toxicity can be ascribed to this therapeutic agent. It is true that recently Lyons[8] has reported an occasional, at present unexplained, sensitization but such a "toxic" effect, if it may be so described, is apparently associated with individual idiosyncrasy rather than with penicillin itself. As pointed out by Lyons, "No significantly harmful effects have been observed."

In testing commercial penicillin for "toxicity" a method has been used which involves the intravenous injection of mice with 100,000 units per kilogram of body weight. Five mice are injected and the product is considered satisfactory if none dies within a forty-eight-hour period of observation. In carrying out routine tests of this nature it soon became obvious that such a test does not measure the "toxicity" of penicillin but rather constitutes a safety test which is capable of eliminating only those products seriously contaminated with substances toxic for mice. In the assay of over 300 lots of penicillin sodium, produced for clinical use by fourteen different manufacturers none has failed to pass the safety test. Similarly, commercially produced penicillin calcium and penicillin ammonium were found to be satisfactory when tested by the above method. A commercial preparation of penicillin magnesium, however, failed to pass the safety test, but in this instance the toxicity, as described later in this report, was shown to be due to the cation rather than to penicillin itself.

In order to determine the acute toxicity of commercial penicillin sodium, the material remaining from each lot after assay, which includes tests for sterility, potency, moisture content, safety, and pyrogens, was injected intravenously into mice in increasing dosages. In some instances it was not possible to establish the "toxic level" of units with individual lots because of lack of material. However, it was possible to establish a "pattern" of the acute toxicity of each manufacturer's product by studying several lots, either indi-

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vidually or pooled. At least three mice were injected at each level of units tested and repeat tests were made at the dose found to be lethal to the extent of the material available for testing.

TABLE I

ACUTE TOXICITY OF PENICILLIN SODIUM IN MICE

MANUFACTURER	NO. OF LOTS TESTED	TOXIC DOSE UNITS 20 GRAM MOUSE*	APPROXIMATE RANGE UNITS, MG.
A	12	3,500- 4,500	100-275
B	6	5,000- 8,000	16-120
C	11	7,000- 9,000	130-320
D	7	8,000-18,000	100-346
E	13	10,000-16,000	140-750
F	8	10,000-16,000	140-330
G	7	12,000-14,000	300-400
H	11†	15,000-18,000	200-500
	5†	8,500- 9,500	134-160
I	15	13,000-21,000	250-540
J	12	25,000-32,000	500-500

*All mice observed for 18 hours.

†Recent lots of this manufacturer's product have shown a lower potency and correspondingly higher toxicity.

In Table I are given the number of units of penicillin sodium which consistently caused death of 20-gram mice when injected intravenously. There is a marked difference in the number of units found to be invariably lethal for mice with the various manufacturers' products. The product of manufacturer A in a study of 12 lots consistently killed 20-gram mice in doses of from 3,500 to 4,500 units, while the product of J consistently killed mice at 25,000- to 32,000-unit dosages. It is of interest that an occasional lot of manufacturer J's product failed to kill mice when injected in 30,000- to 35,000-unit doses in 20-gram mice, while manufacturer A's product has never been found to be innocuous in doses greater than 4,500 units per 20-gram mouse. The toxicity of the penicillin sodium produced by manufacturers B to I, inclusive, falls between 5,000 and 21,000 units. In studying a number of lots of penicillin from different manufacturers over a period constituting the first three months of commercial production, it has been apparent that in some cases at least there has been a general decrease in the toxicity of the products produced. As an example, manufacturer E, who produced penicillin which was toxic at levels of from 7,000 to 8,000 units per 20-gram mouse in early production is now producing penicillin which is toxic for mice at from 14,000 to 16,000 units per 20-gram mouse. There has been a corresponding change in the potency. In early production the potency was from 200 to 300 units per milligram, while recent lots range from 700 to 1,000 units per milligram. Similarly, eleven lots of penicillin produced by manufacturer H, which assayed 200 to 500 units per milligram, were found to be toxic at 15,000 to 18,000 units per 20-gram mouse, while a more recent group of five lots assaying 134 to 160 units per milligram proved to be toxic in doses of 8,500 to 9,500 units per 20-gram mouse. This manufacturer recently reduced the purity of his preparation by changing his extraction method in order to increase his yield of penicillin.

In studies of six different salts of penicillin it has been shown[9] that the toxicity is due mainly to the cation used in the preparation of these salts rather

than to the penicillin itself. Some evidence of this nature will be given later in this report. In the case of penicillin sodium the cation does not appear to be responsible, particularly with those products found toxic at low unitage. (See Table II.) In a previous study[10] it was demonstrated that there are substances elaborated in the production of penicillin by the autolysis of the penicillium mycelia which are both pyrogenic and toxic for mice. It would appear, therefore, that the toxicity at lower unit levels of some manufacturers' products is associated with their methods of extraction of penicillin from the crude metabolic solution. It is of interest, however, that with manufacturer J's product, which was found to have an L_D-50 in mice (intravenous injection) of 28,000 units, the cation is responsible for the toxicity. This manufacturer's product had an average potency of 408 units per milligram and thus the L_D-50 in milligrams of penicillin sodium was 68.6. Since chemical assay showed the presence of 11.7 per cent Na^+, there were 8.0 mg. Na^+ present at the L_D-50. The L_D-50 of Na^+ of sodium acetate was found to be 7.8 milligrams. It is apparent, therefore, that the toxicity in this relatively atoxic product is primarily due to the cation.

COMPARISON OF THE ACUTE TOXICITY OF A CALCIUM AND A SODIUM SALT OF PENICILLIN

Through the courtesy of manufacturer I it has been possible to study the acute toxicity of a calcium and a sodium salt of penicillin, both prepared commercially from a single master lot of material. The potency of the calcium salt was found to be 310 units per milligram with a moisture content of 2.17 per cent, while that of the sodium salt was 332 units per milligram, with a moisture content of 2.0 per cent. Chemical assay of these salts showed the presence of 8.16 per cent Ca^{++} and 9.97 per cent Na^+. To determine the acute toxicity of these two salts at least three mice were injected intravenously in the tail veins with increasing amounts of each salt until a level was reached at which the product was found to be no longer innocuous. The calcium salt was lethal for 20-gram mice in doses of 3,500 units, while a dose of 21,000 units of the sodium salt was required to bring about the same result. From a clinical standpoint this marked difference in toxicity of the sodium and calcium salts may have little significance since on a weight basis 3,500 units of the calcium salt would be equivalent to a single injection of over ten million units in a 60-kilogram man.

On the basis of potency it is obvious that the greater toxicity of the calcium salt cannot be associated with penicillin as such. During the preparation of the salts they had equal opportunity of picking up, from the master lot, substances toxic for mice. To what degree this occurred should be dependent upon their individual combining power with such substances. It would appear, however, that the demonstrated greater toxicity of the calcium salt is not the result of its selective combining power with toxic organic material in the master lot of penicillin since its purity (310 units per milligram) is not much less than the purity of the sodium salt (323 units per milligram).

In attempting to find the cause of the greater toxicity of the penicillin calcium, an L_D-50 was determined in mice for both the penicillin calcium and for calcium acetate. The L_D-50 for the calcium salt of penicillin was found to

be 3,700 units per 20 gram mouse. Since chemical assay showed the penicillin to contain 8.16 per cent Ca^{++} and its potency was found to be 310 units per milligram, the L_D-50 in terms of Ca^{++} is computed to be 0.97 milligrams. The L_D-50 of Ca^{++} of calcium acetate was found to be 1.03 mg. per 20-gram mouse. Therefore, the toxicity of the penicillin calcium is primarily due to the cation used in its preparation, and little, if any, toxicity can be ascribed to organic contaminants or to penicillin itself. Similar tests were made with penicillin sodium and with sodium acetate. In this case the sodium salt had a lethal dose of 21,000 units, a potency as noted above of 323 units per milligram, and it contained 9.97 per cent Na^{+}. The number of milligrams Na^{+} at the lethal dose was therefore 6.31. Since the lethal dose of Na^{+} as sodium acetate was found to be 6.7 mg., it appears that the cation was largely responsible for the toxicity of this sample of penicillin sodium.

TABLE II

THE TOXICITY OF THREE SALTS OF PENICILLIN VS. THE TOXICITY OF THE CORRESPONDING ACETATES

MICE	UNITS PER 20 GM.	MG. PER 20 GM.	MORTALITY RATIO*	MICE	MG. PER 20 GM.	EQUIV. UNITS PENICILLIN PER 20 GM.	MORTALITY RATIO*
	Penicillin Sodium				*Sodium Acetate*		
6	3,500	2.97	0/6	6	6.30	7,500	0/6
6	4,000	3.36	4/6	6	6.70	7,900	2/6
6	4,500	3.78	4/6	6	7.60	9,000	3/6
	Penicillin Magnesium				*Magnesium Acetate*		
6	600	0.276	0/6	9	0.267	580	0/9
6	700	0.322	2/6	7	0.312	670	2/7
6	800	0.367	5/6	6	0.367	798	3/6
	Penicillin Ammonium				*Ammonium Acetate*		
6	4,500	1.23	0/6	6	1.50	5,500	0/6
3	4,700	1.32	2/3	6	1.80	6,600	2/6
6	5,500	1.50	2/6	6	2.10	7,700	4/6
6	6,000	1.64	6/6				

*48-hour observations. Mortality ratio: Ratio of number of mice dying to total number injected.

COMPARISON OF THE ACUTE TOXICITIES OF THE SODIUM, MAGNESIUM, AND AMMONIUM SALTS OF PENICILLIN WITH SODIUM, MAGNESIUM, AND AMMONIUM ACETATES

In view of the results obtained above with a relatively atoxic lot of penicillin sodium, a comparison was made of a more toxic lot of penicillin sodium with sodium acetate. Since both penicillin magnesium and penicillin ammonium have been recommended for clinical trial, similar comparisons were made also with these two preparations. All three penicillin samples tested were commercial preparations. The tests were carried out in a manner similar to that recorded above and the results are given in Table II, where it will be noted that with the sample of penicillin sodium tested, 4,000 units caused death in mice. Since this product contained 84 mg. of Na^{+} per 100,000 units, there were 3.36 mg. of Na^{+} at the 4,000-unit dose. When comparison is made with sodium acetate it will be noted that the lethal dose of Na^{+} of sodium acetate is 6.7 milligrams. Therefore, the toxicity of this sample of penicillin sodium is not due to any great extent to the cation, but rather to either penicillin itself or, more

likely, to organic contaminants resulting from the process of extraction, all of which can be removed by proper extraction methods. The results obtained with this sample of penicillin sodium are in marked contrast to those obtained on the sample of penicillin sodium referred to above which had a lethal dose of 21,000 units. It is of interest that the penicillin sodium referred to in Table II produces marked local reactions in the doses used clinically.

The penicillin magnesium preparation is extremely toxic in comparison to penicillin sodium or penicillin calcium. In the routine safety test, which consists of the injection of five 20-gram mice with 2,000 units each, all animals died within a few seconds, and it was not until the product had been diluted approximately 1:3 (to 600 units) that the preparation became innocuous. It should be pointed out, however, that the penicillin magnesium was a low-potency material (70 units per milligram). The L_D-50 of this sample of penicillin magnesium was found to be 710 units per 20-gram mouse. Since the potency of this product was 70 units per milligram and it contained 3.22 per cent Mg^{++}, the number of milligrams of Mg^{++} at the L_D-50 is therefore 0.325 milligrams. The L_D-50 dose of Mg^{++} of magnesium acetate was found to be 0.38 milligrams. In the case of this preparation of penicillin magnesium, the toxicity of the product is primarily associated with the cation. Another sample of penicillin magnesium, prepared in these laboratories and having a potency of 1,028 units per milligram, was found to have an L_D-50 for mice of 7,600 units. However, again with this high potency preparation, the cation appears to be responsible for the toxicity. Since the L_D-50 was 7,600 units and the potency of the material 1,028 units per milligram, the L_D-50 in milligrams of penicillin is 7.3 milligrams. Chemical assay showed the presence of 4.23 per cent of the cation. Therefore, at the L_D-50 there were present 0.31 mg. of Mg^{++}, which corresponds quite closely to the milligrams of Mg^{++} present (0.38) at the L_D-50 of magnesium acetate. The results obtained with both high and low potency preparations of penicillin magnesium tend to question the clinical use of such material particularly for intrathecal injections.

The lethal dose of penicillin ammonium is approximately 4,700 units (Table II). At this lethal dose there were found present by chemical analysis 1.32 mg. of NH_4^+. By comparison, 1.8 mg. NH_4^+ were found to be present at the lethal dose of ammonium acetate. In the case of penicillin ammonium, the cation, although not primarily responsible for the toxicity of this preparation, probably contributed substantially to the toxic effect. The commercial preparation of penicillin ammonium was relatively insoluble in water, except in very low concentrations. When this preparation was dissolved in water at concentrations of 4,000 units per cubic centimeter a precipitate resulted which could not be dissolved even with the application of heat. The precipitate, however, contained little or no potency, since assay of the supernatant fluid indicated that all of the penicillin was in solution. In performing the tests given in Table II the penicillin ammonium was dissolved in water, the solution centrifuged, and the precipitate discarded before injection of the mice. However, the penicillin solution, on standing for a relatively short time, reprecipitated and it was not possible at any time during the study completely to clarify it. It is quite possible that this precipitate contributed to the toxicity of this

penicillin preparation. In any case it would appear unlikely that commercial penicillin ammonium in its present state of purity is satisfactory for clinical use in man.

SUMMARY AND CONCLUSIONS

A study of over 300 lots of penicillin sodium produced by fourteen manufacturers showed all to pass the mouse safety test.

There is a wide variation in the acute toxicity of different manufacturers' products. Some products are lethal for mice in concentrations of 3,500 to 5,000 units while one manufacturer is producing penicillin sodium lethal only at doses of 25,000 to 32,000 units.

A comparison of commercial samples of penicillin calcium and penicillin sodium made from a single master lot showed the calcium salt to be by far the more toxic preparation. The greater toxicity of the calcium over the sodium salt of penicillin should not discourage its clinical use in man. The toxicity of the penicillin calcium is primarily due to the cation.

The toxicity of high potency samples of penicillin sodium and high or low potency samples of penicillin magnesium is primarily due to the cation used in producing these preparations. Similarly, the cation contributes substantially to the toxicity of penicillin ammonium.

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THE VOLATILE COMPONENTS OF WHITE BREAD PREPARED BY A PRE-FERMENT METHOD[1]

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ABSTRACT

Investigation of the volatile components of bread prepared by a pre-ferment technique resulted in rigorous identification of ethanol, n-propanol, isobutanol, isoamyl alcohol, acetoin, furfural, acetic acid, and ethyl acetate. On the basis of gas chromatographic retention data, propanal, 2-butenal, 2-ethylhexanal, acetone, diacetyl, and 2-methylbutanol-1 were tentatively identified. On the basis of retention data from both thin-layer and flash-exchange chromatography, formaldehyde, acetaldehyde, propanal, n-butanal, isobutanal, n-pentanal, 3-methylbutanal, acetone, 2-butanone, 2-pentanone, and 2-hexanone were tentatively identified. Evidence was obtained for the presence of approximately 17 completely unknown trace components in addition to those listed above. Modification of dough composition by addition of 1,000 p.p.m. of L-proline caused no detectable change in composition of volatile components, in spite of the fact that proline-treated bread was preferred on the basis of its aroma. Odor concentrates prepared from aqueous distillates amounted (ethanol-free basis) to 3-5 p.p.m. of the control bread, and to 8-10 p.p.m. of the proline-treated bread. Components of the odor concentrates were in all cases those listed above. The five alcohols, acetoin, furfural, and acetic acid were by far the major components. Carbonyl compounds were trace components.

Identification of the volatile components of fresh bread has been the goal of many investigations (1–6). Volatile components of fresh white bread prepared by conventional procedures have been shown by Visser't Hooft and deLeeuw (7), Baker *et al.* (8), Wiseblatt and Kohn (9), and Ng, Reed, and Pence (10) to be ethanol, formaldehyde, acetaldehyde, 2-butenal, 2-methylbutanal, n-hexanal, 2-ethylhexanal, furfural, pyruvaldehyde, 2-propanone, 2-butanone, 2-hexanone, 3-heptanone, diacetyl, acetoin, ethyl pyruvate, ethyl levulinate, acetic acid, n-butanoic acid, and 3-methylbutanoic acid. n-Pentanal and 2-methylpropanal have been identified in oven vapors and are presumably volatile bread components. These results were obtained by isolation and separation of single groups of compounds, i.e., acids, alcohols, or 2,4-dinitrophenylhydrazones (2,4-DNPH's) of carbonyl compounds.

Much interest has also been shown in components (both nonvolatile and volatile) of pre-ferments (11–16) and of doughs prepared from pre-ferments (17). Acetic and lactic acids and ethyl acetate were measured

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(11), as were ethanol, total carbonyls, and total organic acids (14,15) in pre-ferments. With the exception of the work of Smith and Coffman (12), who isolated and identified a variety of neutral components of pre-ferments, all other investigations (13,16) were directed toward isolation and separation of carbonyl compounds by formation and separation of 2,4-DNPH's.

This paper describes an investigation of the volatile components of fresh white bread prepared by a pre-ferment method.

To determine the effect of modification of pre-ferment dough composition on the identity and quantity of these volatile components, bread to which L-proline had been added, and which was preferred on the basis of its aroma, was also studied. Since other investigators (2) of bread prepared by the pre-ferment method had isolated only the carbonyl components by 2,4-DNPH formation, it was considered of value to attempt to isolate all volatile bread components in an odor concentrate, to separate them by gas chromatography, and to identify the individual substances thus obtained.

Materials and Methods

Bread-Baking. Bread was prepared by standardized pre-ferment procedure using the formulas (18) given in Table I. The pre-ferment

TABLE I
COMPOSITION OF PRE-FERMENT AND DOUGH

Ingredients	Quantity	Ingredients	Quantity
	g.		g.
Pre-ferment		Dough	
Water (sufficient for 1 liter final volume)		Flour[a]	700
Sucrose	66.0	Sucrose	42
Yeast	44.0	Shortening	21
Sodium chloride	11.0	Sodium chloride	10.5
Diammonium hydrogen phosphate	4.0	Yeast	7
Calcium carbonate	1.0	Pre-ferment solution (ml.)	520
Potassium sulfate	1.0	Water (ml.)	128[b]
Magnesium sulfate	0.5		
Potassium bromate	0.06	Total weight, 1,228.5 g.	
Potassium iodate	0.02		

[a] At 11% moisture.
[b] For 64% absorption.

liquid was stirred and maintained at 86° ± 1°F. for 4 hr. Appropriate aliquots of the pre-ferment broth and an aqueous suspension of yeast, and of solutions of sucrose and sodium chloride, were added in that order to the flour and mixed. The dough was proofed at 90°F. and 90% relative humidity and baked at 425° ± 2°F. for 25 min.

Dough composition was modified by addition of an arbitrarily chosen known amount of the following compounds. L-Arginine, gliadin, casein, and lactalbumin were added to the flour and mixed well before addition of the pre-ferment liquid. DL-Alanine, L-alanine, L-histidine, L-proline, L-leucine, DL-valine, L-valine, maltose, and lactose were added in solution after the pre-ferment was added. Preliminary work indicated that L-leucine added at a level of 500 p.p.m. or 50 mg./100 g. of dough weight could be detected by the panel. All other amino acids were arbitrarily added at 1,000 p.p.m. or 100 mg./100 g. dough weight. Maltose (1.79 g./100 g. dough), lactose (1.79 g./100 g. dough), casein (1.22 g./100 g. dough), lactalbumin (0.24 g./100 g. dough), and gliadin (1.22 g./100 g. dough) were added at levels approximating those which would be found if nonfat dry milk had been used in the dough formula. Amounts were calculated on the basis of the composition of nonfat dry milk (19) and amounts used in the ADMI Pre-ferment Formula (18).

Sensory Evaluation of Bread. Each batch was allowed to cool and was sliced, and the slices were separated into portions of crust and of crumb. Samples of crust and of crumb from control and additive-treated batches were compared in triangle tests. Evaluations were carried out as soon after completion of baking as possible (within 4 hr.). Panel members were students or staff members of the Department who were accustomed to taking part in sensory evaluations. The odor and taste of samples of crust and crumb were evaluated separately. Judges were asked which sample was (or samples were) preferred. Differences significant (20) at 5% level or less were noted.

Chemical Investigation of Volatile Components of Bread. Preparation of Flavor Distillates. Preparation of distillates which truly represented bread flavor and aroma depended upon removal under mild conditions of all volatile flavor and aroma components from as large a quantity of bread as possible. The procedure developed is similar to that described by Wiseblatt and Kohn (9).

Freshly baked warm bread (approximately 13 lb.) was sliced mechanically and placed on perforated aluminum trays inside an 18 by 18 by 1/8-in. pressure cooker. The top of the cooker was fitted with a vacuum gage and an outlet which led through Tygon[3] tubing and a condenser to a series of traps, a McLeod gage, a manostat, and finally to an efficient vacuum pump. The condenser was cooled with cold (50 ± 2°F.) ethanol. A large trap (3-liter) was immersed in a dry ice-ethanol bath, three additional traps (1-liter) were chilled in dry ice-

[3] Reference to a company or product by name does not imply approval or recommendation of the product by the Department of Agriculture.

ethanol, and three traps were chilled in liquid nitrogen. All connections were ball-and-socket glass joints except for the 6-in. length of Tygon tubing.

Immediately after the bread was placed in it, the cooker was closed, steam was passed through copper coils lining it, and distillation was carried out at 14–28 mm. Hg. Temperature within the cooker did not rise above 30°C. After about 8 hr. approximately 40% of the crumb had become dry. Pieces which still contained moisture were moved to the upper trays and distillation was continued for 8 more hr. until all the crumb was dry; it was ground and placed in the cooker, and distillation was carried out at 1 mm. Hg for approximately 1 hr. The resulting dry residue was odorless. However, typical bread odor could be regenerated in it by the addition of water.

The oxidizable carbon content (21) of the various distillates provided an index of the amount of organic matter present in each condensate and, in addition, allowed a rough comparison of the total yield of volatile substances obtained from each bread preparation. Almost no liquid condensate was collected in the liquid nitrogen traps during any of the distillations. The contents of each of these traps were treated with 10 ml. of 2*M* 2,4-dinitrophenylhydrazine hydrochloride.

The ethanol content of the distillates was determined by gas chromatography of 1-μliter samples on a Perkin-Elmer F. column (tetraethylene glycol dimethyl ether), 2 meters × 1/4 in., at 61°C. A standard curve prepared by plotting ethanol peak height against ethanol concentration for a number of known solutions of aqueous ethanol allowed analysis of the distillates. As noted by Wiseblatt and Kohn (9) in their work, colorimetric estimations of total carbonyls and esters in the distillates were not reliable.

Isolation of Odor Concentrates. Odor concentrates were obtained by saturation of the various distillates with sodium chloride, extraction with diethyl ether (1 liter ether:1 liter distillate) and concentration of the dried ether extract. Concentration was carried out in preparations 2, 3, and 4 by nitrogen entrainment and in subsequent preparations by fractional distillation. In the first case, the ether removed was collected in a solution of 2*M* 2,4-dinitrophenylhydrazine hydrochloride. Formation of a precipitate indicated that losses of carbonyls had occurred.

Concentration of ether extracts by distillation in a 0.5 × 9-in. column packed with glass helices was accomplished with minimal losses as determined by intermittent gas-chromatographic analysis of the ether recovered, on a column 2 m. × 2 mm. i.d., of 5% β,β'-oxy-

dipropylnitrile on Gas Chrom A (100–120-mesh) at 46°C. in a Research Specialties Model 60-1 Flame Ionization chromatograph. The presence of ethanol was always detected. During the last stages of concentration trace amounts of components which had retention times in agreement with those of n-propanol, isobutanol, and isoamyl alcohol were detected in the ether distillate. These losses are not believed to have been significant.

Gas-Chromatographic Separation of the Components of Bread Odor Concentrates. A column 2 m. × 4 mm. i.d. of 20% Quadrol (tetrakis (2-hydroxypropyl) ethylene diamine) on firebrick (42–60-mesh), operated at 103°C., at a flow rate of 75 ml./min. and a helium pressure of 10 p.s.i.g., gave the best separation of the bread odor concentrates. The chromatograph used was constructed in our laboratory. Its detector contained matched 100,000-ohm thermistors.

Since the Quadrol column was alkaline and would irreversibly absorb acidic components, the odor concentrates were also separated on a column 2 m. × 8 mm. i.d. of 20% Ucon LB 1715 on chromosorb (30–60-mesh), operated at 101°C., flow rate 100 ml./min., helium pressure 4 p.s.i.g. A column 2 m. × 8 mm. i.d. of 20% DEGS (diethyleneglycol succinate) on acid-washed chromosorb (60–65 mesh), maintained at 60°C., flow rate 150 ml./min., helium gas pressure 10 p.s.i.g., was used to gain evidence for 2-methylbutanol-1 in the odor concentrates. It was known to separate this compound from isoamyl alcohol at these conditions. Separation of odor concentrates was also carried out on a column 2 m. × 2 mm. i.d. of 5% β,β'-oxydipropylnitrile (OPN) on Gas Chrom A (100–120-mesh) at 43°C. in a Research Specialties Model 60-1 Flame Ionization chromatograph.

Identification of the Components of Odor Concentrates. The fractions obtained by separation on the above three columns were trapped at the column exit in U-tubes chilled in liquid nitrogen. Their identification was accomplished if their infrared spectra were identical with spectra of known pure reference compounds and if their retention data were in agreement with those of the reference compounds. Retention times were in all cases calculated relative to isobutanol. Reference spectra were obtained from known compounds after purification by gas chromatography. To obtain a large enough quantity of any fraction (except the ethanol fraction) for spectrum analysis it was necessary to collect from at least three successive 40-μliter samples of concentrate. Even so, pure liquid spectra were not obtainable and it was necessary to add carbon tetrachloride (about 1 μliter) to fill the infrared cell. Spectra were obtained with a Beckman IR-5 spectrophotometer fitted with a 5X KBr beam condenser. Type D sodium chloride cells

(Connecticut Instrument Co.) having 0.05-mm. nominal path length were used.

The purity of individual trapped fractions was determined by rechromatography on either a column 2 m. × 2 mm. i.d. of 1% Ucon 50 HB 2000 on 100- to 120-mesh Gas Chrom A at about 36°C. or on the 5% β,β'-OPN column at 50°C. Flame ionization detectors in an Aerograph Model A-500B and a Research Specialties Model 60-1 were used. Columns and conditions were selected on the basis of their ability to separate compounds shown by other investigators (8,9,10) to occur in bread. With the exception of ethanol, which was easily trapped in large volumes, the carbon tetrachloride solutions used for infrared spectrum determinations of each fraction were rechromatographed.

Investigation of Acidic Constituents of Bread-Odor Distillates. Since acidic constituents might not have been completely removed by ether extraction, the odor distillates were lyophilized (to remove sodium chloride) and the resulting distillates made just alkaline. The alkaline solutions were lyophilized and the residue was isolated. Free acids were regenerated from sodium salts by the procedure of Hunter, Ng, and Pence (14) and separated on a column 2 m. × 4 mm. i.d. of 5% LAC IR-296 on acid-washed firebrick (60- to 80-mesh) at 125°C. The procedure was established by separation of a known mixture (normal C_2-C_5 and iso-C_4-C_5) of free acids and of the acids regenerated from their salts.

Investigation of Carbonyl Components of Bread-Odor Distillates. Immediately after completion of the distillations, 2,4-DNPH derivatives were prepared from the condensates collected in the traps chilled with dry ice-ethanol and liquid nitrogen. 2,4-DNPH's were also prepared from a 1-ml. aliquot of each of the ether-soluble odor concentrates and from the ether removed during concentration of the ether extracts, as were 2,4-DNPH derivatives of authentic samples of known bread components.

Separation by Thin-Layer Chromatography. The derivatives (5 μliters of a 0.1% (w/v) chloroform solution) of known carbonyls and of the unknown bread derivative mixtures were separated on a 0.01-in. layer of Silica Gel G (Brinkmann Instruments, Inc.) in water-saturated benzene (22). R_f values were determined relative to formaldehyde-2,4-DNPH. The reproducibility of separation of known derivatives varied from 2.43 to 5.01%, on the basis of 21 runs. Reproducibility of separation (17 runs) of bread mixtures varied from 1.52 to 4.38%.

Gas-Chromatographic Separation of Carbonyl Compounds Regenerated from 2,4-DNPH Derivatives. Carbonyls were regenerated according to Ralls' procedure (23,24) from selected spots isolated on the

chromatoplates from bread fractions. A vial containing the reaction mixture was heated in an oil bath at 250°C. for 10–15 sec. and a 500-μliter vapor sample of its head gas was separated on a column 6 ft. × 1/8 in. of 1% Ucon 50 HB-2000 on 100- to 120-mesh Gas Chrom A at 31°C. An Aerograph A-500-B flame ionization detector was used. On the basis of relative peak areas, the carbonyl compounds could easily be selected from peaks caused by artifacts.

Results and Discussion

Sensory evaluation of control bread and of breads having modified dough composition showed that though differences were noted in a number of cases (Table II), only the addition of L-proline improved

TABLE II

SENSORY EVALUATION IN A TRIANGLE TEST OF BREAD PREPARED BY MODIFICATION OF PRE-FERMENT DOUGH COMPOSITION

Test No. and Additive[a]	Number of Correct Judgments	Number of Incorrect Judgments	Level of Significance	Preference
1. DL-alanine (100 mg.) Crust: Odor	26	12	<0.001	18 control 8 DL-alanine
Taste	26	12	<0.001	16 control 9 DL-alanine
2. L-arginine (100 mg.) Crumb: Odor	21	16	0.01	11 control 10 L-arginine
3. L-histidine (100 mg.) Crumb: Taste	16	14	0.05	8 control 5 L-histidine
4. L-proline (100 mg.) Crumb: Odor	19	19	0.05	4 control 13 L-proline
Crust: Odor	23	15	0.001	7 control 15 L-proline
Taste	23	15	0.001	12 control 11 L-proline
5. L-leucine (50 mg.) Crumb: Odor	22	21	0.03	2 control 1 L-leucine
6. L-valine (100 mg.) Crust: Odor	20	16	0.01	16 control 3 L-valine
Taste	19	17	0.03	15 control 2 L-valine
7. Casein (1.22 g.) Crumb: Odor	9	5	0.05	6 control 3 casein
8. Gliadin (1.22 g.) Crumb: Odor	10	5	0.01	9 control 1 gliadin
Taste	10	5	0.01	7 control 2 gliadin
Crust: Taste	9	6	0.05	5 control 4 gliadin

[a] Amount per 100 g. dough.

bread aroma. The amount of proline added (868 μmoles/100 g. or 100 mg./100 g. of dough) was very large, both from a practical standpoint and relative to the quantity of free proline normally found (9.60 μmoles/100 g. of dough) in fermented bread doughs (25). However, since a primary goal of the research was to determine whether improved flavor brought about by modification of dough composition would be reflected in the chemical composition of volatile components, the large quantity of proline used might be an advantage. Yields of volatile components might be increased.

Distillation of sliced fresh bread yielded aqueous distillates which exhibited characteristic (but not fresh) bread aroma. Table III gives

TABLE III

SUMMARY OF PREPARATIONS OF BREAD ODOR DISTILLATES AND CONCENTRATES

No.	Bread	Distillates				Odor Concentrates		
		Volume	Oxidizable Carbon Content	Ethanol Concentration		Weight	Weight[b]	Yield[b]
				Found	Theory[a]			
	lb.	*ml.*	*g./lb.*	*g./lb.*	*g./lb.*	*g.*	*mg.*	*p.p.m.*
2	13.7	1,780	0.80	1.6	1.6	1.80	18	3
3	13.8	1,585	0.72	1.5	1.5	2.70	27	5
4	13.0	1,624	0.85	~1.8	1.8	2.22	22	3
5	12.6	1,730	0.87	2.0	1.8	3.54	35	~ 5
6[c]	12.8	1,780	1.06	2.7	2.3	6.08	60	~10
7[c]	12.6	1,704	1.03	2.2	2.1	4.80	47	~ 8

[a] The theoretical ethanol concentration is the amount of ethanol calculated as being present if all the oxidizable carbon found is assumed to be in the form of ethanol.
[b] Reported on an ethanol- and ether-free basis to emphasize the minute quantity of bread-flavor components present. Ether is present as a residue from ether extraction of the aqueous distillates.
[c] Proline added.

a summary of bread distillations. The oxidizable carbon content of the total distillates from identical control bread preparations (4 and 5, Table III) was essentially the same. Preparations 6 and 7 in which the dough had been modified by addition of L-proline at 1,000 p.p.m. of dough weight also yielded distillates having reproducible oxidizable carbon content. Their ethanol content in g. per lb. of bread is given in Table III, along with their theoretical ethanol content (calculated on the assumption that all the oxidizable carbon found was present in ethanol). In all cases the oxidizable material detected was accounted for as ethanol (Table III), indicating that the flavor components were present in minute quantities.

Gas-chromatographic analysis of the odor concentrates showed that ethanol and diethyl ether were by far the largest components present. On the basis of relative peak areas, these accounted for approximately 99% of each of the various concentrates. The weights of all odor isolates obtained (calculated on an ethanol- and ether-free basis) are

shown in Table III, along with the yields in which they were obtained from bread. They represented from 3 to 5 p.p.m. of control bread (preparations 2 through 5) and 8 to 10 p.p.m. of bread to which proline had been added (preparations 6 and 7). Informal sensory evaluation indicated that they exhibited typical (but not fresh) bread aroma.

Gas-chromatographic separation of the odor concentrates on a 20% Quadrol column showed (Fig. 1) the presence of only six components

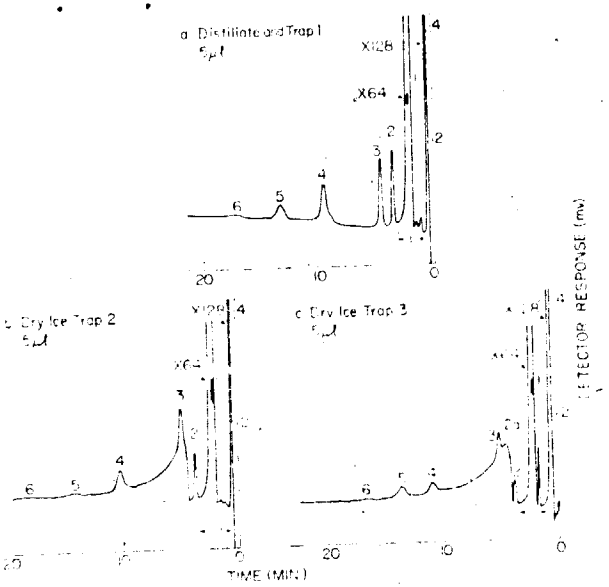

Fig. 1. Chromatograms of bread odor concentrates (prep. 5) on a 20% Quadrol column at 103°C. The unnumbered peak is diethyl ether. Peak 1, ethanol; 2, n-propanol; 2a, water; 3, isobutanol; 4, isoamyl alcohol; 5, acetoin; 6, furfural.

in addition to two trace substances detected in fraction 1. The isolates from the dry-ice traps (curves b and c, Fig. 1) contained an additional component (fraction 2a) which was later proved to be water. Similar chromatograms were obtained from analogous odor concentrates derived from all bread preparations. On the basis of the identity of their infrared spectra and retention data with those of authentic reference compounds, the peaks shown were proved in all the isolates to contain n-propanol (fraction 2), isobutanol (fraction 3), isoamyl alcohol and possibly 2-methylbutanol-1 (fraction 4), acetoin (fraction 5), and furfural (fraction 6). In all cases fraction 1 contained ethanol. Separation of the odor concentrates on a 20% Ucon LB 1715 column allowed isolation and identification of acetic acid which was not eluted from the alkaline Quadrol column. The presence of a shoulder on the isoamyl

alcohol fraction (number 4) on the Quadrol column indicated that this fraction was impure. Since its infrared spectrum was identical with the spectrum of known isoamyl alcohol, the impurity was concluded to be very similar in structure to isoamyl alcohol. This suggested the presence of 2-methylbutanol-1. The bread odor concentrates were therefore separated on a 20% DEGS column. On the basis of retention data, a fraction corresponding to 2-methylbutanol-1 was obtained, but not in large enough quantity to allow a definitive spectrum to be recorded. It is probable that 2-methylbutanol-1 was present. Use of the DEGS column also allowed identification of ethyl acetate, since the infrared spectrum of a small fraction eluted before ethanol was identical with that of authentic ethyl acetate.

The small number of components isolated from the above three columns was disappointing. However, it is not illogical if one reviews (Fig. 2) the separation obtained by means of the 5% β,β'-OPN column

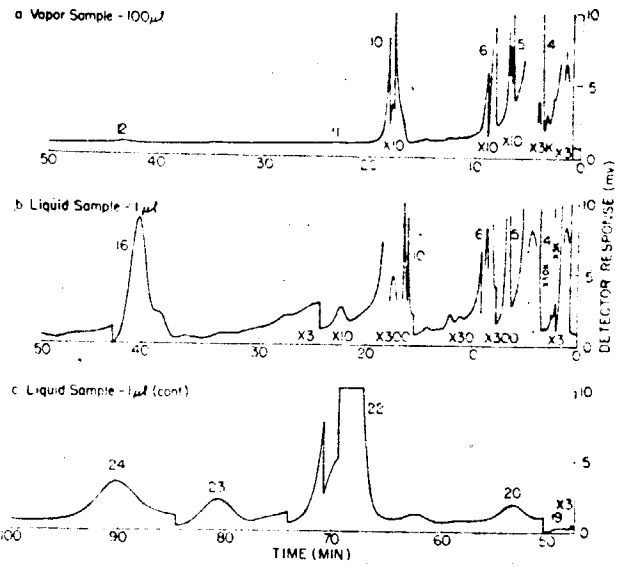

Fig. 2. Chromatograms of a bread odor concentrate (prep. 4) on a 5% β,β'-oxydipropylnitrile column at 43°C. (vapor vs. liquid samples). Peak 1, residual diethyl ether; 4, ethanol; 5, n-propanol; 6, isobutanol; 10, isoamyl alcohol; 22, acetoin (?).

and a flame ionization detector. This chromatogram, and the fact that all the odor concentrates, irrespective of the preparation or cold trap from which they were derived, gave a chromatogram qualitatively similar to it, showed that only six major components were present.

Other substances were present in quantity too small to be detected by the conventional gas chromatographs necessarily used for separation and subsequent collection of individual fractions.

The major peaks (1, 5, 6, and 10 in both the vapor and liquid sample in Fig. 2) were ethanol, n-propanol, isobutanol, and isoamyl alcohol respectively. Of the approximately 11 additional substances detected in the liquid sample, only peak 22 could be tentatively identified as acetoin. Compounds identified by other investigators as components of bread would, with the exception of furfural, be eluted within the first 20 min. of the chromatograms. Furfural remains on the column at the conditions used. The chromatogram of the liquid sample shown in Fig. 2, b, thus provided evidence for the presence of at least 11 components not previously detected in bread. It is possible that additional substances would have been detected if the separation had been temperature-programmed. Though known to be a bread component, hydroxymethylfurfural was not expected to be found, since it is not volatile enough to have been distilled from the bread. Alcohols are by far the major constituents of the volatile components of bread. This suggests that man's odor receptors may be relatively insensitive to alcohols and very sensitive to the unknown and minor bread-odor components.

An example of the results of rechromatography of individual fractions is given in Fig. 3. The infrared spectrum of the fraction was

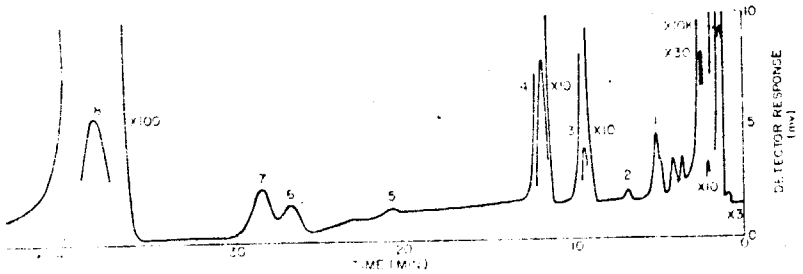

Fig. 3. Rechromatography of fraction 5 (prep. 4) trapped from a 20% Quadrol column on a 5% β,β'-oxydipropylnitrile column. The initial two large peaks are carbon tetrachloride (from the IR spectrum solution) and ethanol. Peak 1 is probably isobutanol; peak 3, isoamyl alcohol; peak 8, acetoin. The rest are unknown.

identical with that of an authentic sample of acetoin. However, rechromatography showed nine additional trace components. On the basis of retention data, the initial two peaks were carbon tetrachloride and ethanol. Peak 1 was apparently isobutanol and peak 3, isoamyl alcohol. Detection of trace quantities of preceding fractions was common. Peak 4 may be 2-ethylhexanal. The identity of the remaining constituents is unknown.

On the basis of results of this nature, tentative evidence was obtained for the presence of very small quantities of acetone, 2-butenal, diacetyl, propanal, 2-ethylhexanal, and approximately 17 completely unknown trace components. Acetic acid was the only acidic component detected, though the analytical method used (14) was capable (if the substances were present in sufficient quantity) of detecting the n-C_2-C_8 and iso-C_4-C_5 acids. Lactic and pyruvic acids, known bread constituents, were not expected to be found, since, owing to nonvolatility, they could not be present in the odor concentrates. No significant differences in composition of control and proline-treated bread odor concentrates were found.

Since direct gas chromatography of odor concentrates resulted in identification of only two carbonyl compounds, furfural and acetoin, and since numerous carbonyls were known to be bread components, it was clear that additional compounds should be detected and identified. Separation of 2,4-DNPH's from the odor concentrates, the contents of the traps cooled with dry ice-ethanol and liquid nitrogen, by thin layer chromatography is shown in Fig. 4. Qualitative variations were noted in the carbonyl content of analogous fractions within the control and proline-treated bread preparations as well as between these preparations. Regeneration from selected 2,4-DNPH spots and gas-

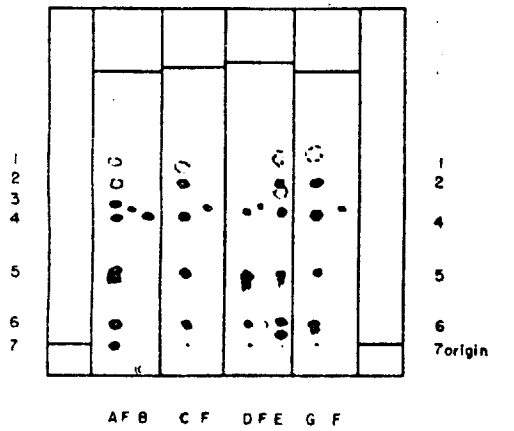

Fig. 4. Thin-layer chromatography of 2,4-DNPH's of bread isolates. A, odor concentrate; B, ether removed during concentration of ether extract; C,D,E, condensates in dry ice-ethanol traps; G, condensate in liquid-nitrogen trap; F, formaldehyde-2,4-DNPH. Spots 1, 2, 3, and 4 were regenerated and gas-chromatographed. Spot 5 is a mixture of acetoin and diacetyl-2,4-DNPH's. Spots 6 and 7 are believed due to 2,4-DNPH reagent.

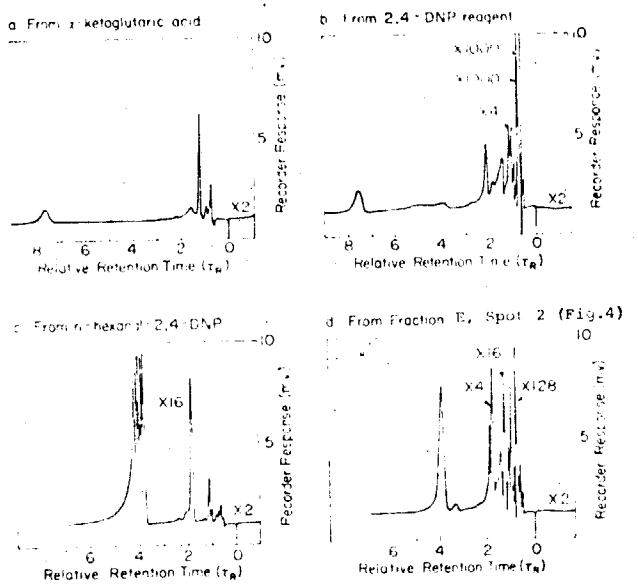

Fig. 5. Gas chromatograms obtained by regeneration of selected 2,4-DNPHs. Column: 1% Ucon 50 HB 2,000; column temperature 35°C.

chromatographic separation of the resulting free carbonyl compounds gave chromatograms typical of the one given in Fig. 5 (curve d). Only peaks which were clearly not derived from decomposition of alpha-ketoglutaric acid or of 2,4-DNPH reagent were considered. Thus the small peak at t_R 3.9 was not recorded as a carbonyl component of the spot. However, the attenuated peaks at t_R 0.811, 1.34, and 1.81 were believed to be bread components. On the basis of R_f values and retention data from thin-layer chromatography and flash-exchange gas chromatography (Table IV) of 2,4-DNPHs, formaldehyde, acetaldehyde, acetone, propanal, n-butanal, isobutanal, n-pentanal, 3-methylbutanal, 2-butanone, 2-pentanone, and 2-hexanone were tentatively identified.

Since chromatography is a separation procedure and not an identification method, the identities proposed above are tentative even though based on retention data from both thin-layer and gas chromatography. The fact that all the above compounds (with the exception of n-propanal, n-butanal, 2-pentanone, and 3-methylbutanal) have been shown by others (4,9,10) to be bread components gave needed support for their presence. The exceptions need further proof of their identity. It is quite probable that 2-methylbutanal was present as well as 3-methylbutanal. In view of the tentative nature of the identifications,

TABLE IV
THIN-LAYER AND FLASH-EXCHANGE CHROMATOGRAPHY OF CERTAIN UNKOWN 2,4-DNPH BREAD FRACTIONS

Fraction[a]	Thin Layer Chromatography: Spot	R_f	Tentative Identity	Flash-Exchange Chromatography: Peak	t_R[b]	Conclusion
E	2	1.37	n-C_4al, iso-C_4al, 2-C_5one, n-C_5al	9	0.811	isobutanal
				8	1.34	?
				11	1.81	2-pentanone
	3	~1.11	C_3al, C_4al, 2-C_4one	1	0.520	?
				3	0.707	propanal
				4	0.747	?
				6	1.12	2-butanone
				7	1.31	?
G	2	1.13	iso-C_4al, n-C_4al, 2-C_5one, n-C_5al, 2-C_6one, 3-methylbutanal, furfural	2	0.614	?
				3	0.734	?
				4	0.827	isobutanal
				5	1.00	n-butanal
				6	1.06	?
				7	1.12	?
				8	1.33	3-methylbutanal
				11	1.83	n-pentanal
				16	3.94	2-hexanone
	4	0.995	C_3al, C_2al, C_3one	2	0.587	acetaldehyde
				3	0.676	formaldehyde
				4	0.787	acetone
				5	1.09	?
				6	1.55	?
				9	2.02	?

[a] From Fig. 4.
[b] Retention time relative to n-butanal.

no significance was attached to qualitative or quantitative differences observed between bread preparations. To our knowledge the presence of n-propanol, isobutanol, isoamyl alcohol, and ethyl acetate in bread has not previously been proved, nor has evidence for the presence of n-butanal, 2-pentanone, or 2-methylbutanol-1 been previously presented. It is logical that such compounds would be present.

Since previous knowledge of the volatile components of bread concerned primarily the nature of carbonyl compounds, it was initially surprising to find them present in such small quantity relative to other organic components. However, the situation is logical if one realizes that the carbonyl components had been isolated, separated, and identified as 2,4-DNPH derivatives. Noncarbonyl compounds were generally not studied in any detail. The fact that Wiseblatt and Kohn (9) did not report acetoin as a volatile bread component, whereas acetoin was found by us to be a major component, is interesting in this regard. The reason may be that the presence of excess 2,4-dinitrophenylhydrazine in Wiseblatt's distillate caused conversion of acetoin-2,4-DNPH to diacetyl-2,4-DNPH. If this did occur, no acetoin would be detected

and 2,4-dinitrophenylaniline would have been formed. Demonstration of its presence in the reaction mixture would support this postulate.

The possibility that 2-ethylhexanal may be artifact and not a bread component must be recognized. It is conceivable that Tygon tubing may have contaminated the condensates with 2-ethylhexanol, which in turn could have been converted to 2-ethylhexanal. It must also be recognized that oxygen from air may have been condensed in the liquid-nitrogen traps and have contributed to artifact production.

Much remains to be learned before the chemistry of bread flavor is understood. The many completely unknown trace components detected in this investigation may contain the missing information.

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METABOLISM OF ACETATE, PROPIONATE, AND N-BUTYRATE IN YOUNG MILK-FED CALVES[1]

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ABSTRACT

The metabolism of acetate-1-C^{14}, propionate-1-C^{14}, and n-butyrate-1-C^{14} was studied in milkfed calves up to 80 days of age. Quantities of each acid calculated to supply one-half of maintenance energy were infused intravenously for 8 hrs. During the infusion, expired air was monitored for $C^{14}O_2$, and venous blood was sampled for the isolation of metabolic intermediates. The expired CO_2 derived from the oxidized portion of the infused acids was between 20 and 30% of total expired CO_2, indicating that the acids could be metabolized in relatively large quantities by young calves. There were no significant differences in rates of metabolism attributable to individual acids or to age. Acetate, propionate, and butyrate appeared to be metabolized largely by established pathways. Acetate and butyrate resulted in increased ketone production, whereas propionate caused a decreased production. Propionate and butyrate contributed about equally, but greater than acetate, to blood glucose labeling. Infusion of all acids resulted in decreased blood glucose levels and increased blood lactate levels. During infusions of acetate and butyrate both the concentration and the specific activity of blood malic acid increased, suggesting the presence of at least a portion of the glyoxylate pathway.

It is well-established that the volatile fatty acids, i.e., acetic, propionic, and butyric, produced by fermentation in the rumen contribute significantly to the energy needs of the adult ruminant animal. Carroll and Hungate (5), for example, estimated that 70% of the total energy requirement of cattle was derived from the metabolism of such acids. Some difference of opinion exists, however, concerning the relative importance of individual acids. In contrast to the adult, volatile fatty acids are not an important source of energy for the newborn ruminant. The rumen is undeveloped and essentially nonfunctional during this stage of life and, hence, there is little or no production of these acids. The ability of the very young ruminant to metabolize volatile fatty acids has not been investigated extensively. Martin et al. (13) fed a purified diet containing salts of volatile fatty acid to young calves. Though the results indicated that such acids could be utilized by animals as young as 3 wk of age, their specific contribution to total energy requirement was not quantitated. Thus, the objectives of the research reported herein were primarily twofold: a) to investigate the ability of the young milk-fed calf at various ages to utilize large quantities of volatile fatty acids, and b) to study the pathways of metabolism that appear to be involved.

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EXPERIMENTAL PROCEDURE

The test subjects in this experiment were male Holstein calves. These animals were obtained at one to three days of age and were fed twice daily throughout the experimental period a diet of whole milk supplemented with vitamins A and D, trace minerals, and chlortetracycline. At 10, 37, and approximately 80 days of age, each calf received an 8 hr intravenous infusion of either acetate-1-C^{14}, propionate-1-C^{14} or n-butyrate-1-C^{14}. Samples of blood, urine, and expired CO_2 were collected throughout the infusion period and for 3 hr thereafter. A total of 11 infusions involving six calves was conducted (Table 1). The infusion of acetate at ten days of age was replicated three times.

On the afternoon prior to an infusion, the calf was placed in a metabolism cage and a

TABLE 1

Schedule of infusions to calves at various ages

Identification no. of calf	Age	Acid infused
	(days)	
1	10	Acetate
4	10	Acetate
5	10	Acetate
3	37	Acetate
2	80	Acetate
1	10	Propionate
5	37	Propionate
3	76	Propionate
3	10	Butyrate
2	37	Butyrate
6	77	Butyrate

polyethylene catheter established in each jugular vein (17), one of the catheters for the infusing of acid and the other for the withdrawal of blood. The total quantity of acid to be infused was adjusted in accordance with live weight to provide a combustible energy value (9) equal to one-half of the digestible energy required during the 8-hr infusion period for maintenance (4). The solutions of propionic and butyric acid were adjusted to pH 7.0 with a 1:1 mixture of NaOH and KOH. Solutions of acetic acid, however, were adjusted only to pH 4.4 (60% neutralization), a step necessary to prevent alkalosis during infusion. The appropriate C^{14}-labeled acid, 400μc, was added subsequently, and the solutions were diluted to final volume of six liters. The rate of infusion was 750 ml per hour.

The usual feeding of milk was withheld on the morning of an infusion. At the start of infusion, a face mask (10) having a three-way valve was placed on the calf. The volume of expired air was measured continuously with a dry-gas meter[4] having an aliquoting device. The aliquot of expired air was passed through a triple NaOH trap to remove the CO_2. Though the above meter is supposed to remove an aliquot of particular size, this was found to vary somewhat. Thus, it was necessary to employ a wet-test gas meter[5] to measure the volume of the aliquot accurately. The meters were read and the CO_2-trap was changed at hourly intervals. The contents of the CO_2-trap were stored at 2 C until subsequent analysis. The face mask was removed briefly at the end of the infusion period, at which time the calf was offered a normal feeding of milk. The mask was replaced after the milk was consumed and the metering of expired air continued until 11 hr after start of the infusion.

Samples of blood, approximately 125 ml, were withdrawn via the catheter at 0, 1, 4, 8, and 11 hrs. Protein-free filtrates were prepared immediately (16) and stored at -15 C for subsequent analysis. Urine was collected continuously and sampled hourly during the entire 11-hr period.

Expired CO_2 was measured by adding 1.0 ml of the solution from the NaOH trap to 0.5 ml of 1 M $BaCl_2$ and titrating with H_2SO_4 under nitrogen to the phenolphthalein endpoint. Expired $C^{14}O_2$ was determined by placing 3 to 5 ml of the solution from the NaOH trap into a 50-ml center-well flask. A quantity of Hyamine[6] hydroxide estimated to represent a 20% excess was added to a small glass shell vial in the center-well of the flask (20). The flasks were sealed and 2 ml of 4 N H_2SO_4 were injected to release the CO_2. The flasks were shaken in a water bath at 37 C for 1 hr, during which time they were protected from sunlight to prevent discoloration of the Hyamine. The shell vial was subsequently removed from the flask and placed immediately into a counting vial containing 15 ml of toluene scintillation mixture and the contents mixed thoroughly. Each sample was counted in a liquid scintillation spectrometer and individual counting efficiency was measured by use of internal standards.

Blood glucose was measured by the Nelson-Somogyi method (16). For radioactivity measurements, the glucose from 25 ml of blood was isolated as the gluconate (2), subsequently tared, dissolved in 3 ml of water, and counted in 10 ml of dioxane scintillation fluid.

The organic acids in 50 ml of blood were separated by the procedure of Ramsey (18). Thymolphthalein was used as an indicator for titrating the acids. For radioactivity measurements, the fractions representing each acid peak were pooled after titration and dried under forced air at room temperature. The residue was dissolved in 1 ml of a 1:1 mixture of ethanol and water. A portion of this solution, 0.7 ml, was placed on a strip of Whatman 3 MM filter paper, 1.0 by 1.5 in. and dried in a stream of warm air. The strip of filter paper containing the organic acid salts was placed in a counting vial containing toluene scintillation fluid for subsequent measurement of C^{14}.

The total amount of C^{14} in urine was determined by placing a 1.0-ml alkaline aliquot on filter paper, as described for organic acids.

[4] Zentral-Werkstatt Gottingen, Busenstrasse 10, Gottingen, West Germany.

[5] Precision Scientific Co., Chicago, Illinois.

[6] Packard Instrument Company, Inc., LaGrange, Illinois.

RESULTS

General Response

The general clinical response of the calves to the infusions was satisfactory. The initiation of infusion and the placing of the face mask on the calf were usually followed by a temporary increase in both rate and total volume of respiration, which usually subsided by the third or fourth hour. The intensity of increased respiration seemed to be related to the temperament of individual calves. Another response was frequent and profuse urination, with an average of 5.2 liters being excreted during the 11-hr collection period. On four occasions, the urine appeared to contain traces of blood, though this did not continue after infusions were completed and could not be related to any ill effects. The milk offered at the end of the infusion was usually consumed readily. In three cases, only part of the milk was consumed, though these calves were eating normally by the following day.

OXIDATION OF INFUSED ACIDS TO CO_2

The extent to which acetate, propionate, and n-butyrate are oxidized to CO_2 is of primary importance in evaluating the ability of the young calf to utilize these acids. Thus, hourly observations on the per cent of expired CO_2 derived from each infused acid are presented in Table 2. In general, these values increased steadily during the first 3 to 4 hr, tended to plateau from the fourth through the eighth hour, and decreased gradually thereafter. The age of the calf seemed to have little or no effect on this pattern. In the absence of marked age trends, hourly means for each acid also are presented in Table 2. There were no marked differences among the three acids, except that propionate made the highest contribution to expired CO_2 during the first 3 hr of the infusion period; whereas, acetate made the greatest contribution after completion of the infusion from the ninth through the eleventh hour. The foregoing observation may be a reflection of differences in the size of the body pools of the acids in question. Thus, if the body pool of acetate is larger than that of propionate, the equilibration of infused acetate with the pool may require a longer period of time than propionate. In summary, results presented in Table 2 show that approximately 20 to 30% of the CO_2 expired from the fourth through the eighth hour was derived from the infused acid, thus indicating that the young milk-fed calf can oxidize an appreciable quantity of acetate, propionate, and n-butyrate.

Since the per cent of expired CO_2 derived from infused acid was maximal and tended to plateau from the fourth through the eighth hour of the infusion, means for this interval were computed (Table 3) and are considered

TABLE 2

Percentage of expired CO_2* by hourly periods, originating from infused acetate, propionate, and butyrate

Infused substance	Calf No.	Calf Age	CO_2 expired at hourly intervals (%) 1	2	3	4	5	6	7	8	9	10	11
		(days)											
Acetate	1	10	5.0	13.5	21.0	23.2	30.9	29.3	35.6	32.7	31.1	15.8	12.1
	4	10	4.8	11.4	17.8	23.0	23.3	18.9	26.4	34.3	15.6	17.7	13.3
	5	10	5.1	11.1	17.1	20.4	27.4	26.0	27.7	25.1	28.3	19.2	14.7
	3	37	6.7	10.8	13.7	17.3	19.7	20.5	21.1	18.0	21.8	19.6	12.8
	2	80	2.7	9.1	14.4	12.8	18.7	19.7	22.5	23.3	23.8	20.1	13.0
		Mean	4.9	11.2	16.8	19.3	24.0	22.9	26.7	26.7	24.1	18.5	13.2
Propionate	2	10	8.8	19.7	23.4	23.7	21.7	24.9	23.6	23.0	18.7	12.1	8.2
	5	37	13.7	12.5	21.0	21.1	19.4	22.5	20.7	20.3	20.0	10.3	
	3	76	6.8	12.3	15.5	17.2	22.3	19.7	25.5	23.7	22.1	12.5	9.3
		Mean	9.7	14.7	19.8	20.5	20.9	22.4	26.0	25.1	20.1	11.5	8.8
Butyrate	3	10	5.5	12.5	22.2	21.3	25.1	32.1	31.0	23.2	16.1	10.9	6.4
	2	37	4.4	10.0	19.4	17.7	23.4	23.1	14.0	28.0	17.5	6.7	6.2
	6	77	4.6	10.2	12.9	14.8	16.4	20.1	20.9	22.1	22.8	18.0	12.6
		Mean	4.8	10.8	18.0	17.8	21.4	24.9	21.7	24.3	18.6	11.7	8.3

* Percentage of expired CO_2 originating from infused acid =

$$\frac{\text{Specific activity of expired } CO_2}{\text{Specific activity of infused acid}} \times \frac{\text{Number of C atoms in infused acid}}{} \times 100.$$

TABLE 3

Summary of mean oxidation of acetate, propionate, and butyrate to CO_2 from the fourth through the eighth hour of infusion period

Infused substance	Calf No.	Calf Age	Per cent of expired CO_2 derived from: Infused acid	Infused plus endogenous acid [a]	Endogenous acid	Per cent of maintenance energy derived from infused acid [b]
		(days)				
Acetate	1	10	30.3	33.8	3.5	32.2
	4	10	25.2	27.0	1.8	32.5
	5	10	25.3	27.8	2.5	31.8
	3	37	19.3	21.5	2.5	29.0
	2	80	19.4	27.2	7.8	29.8
		Mean	23.9	27.5	3.6	31.1
Propionate	2	10	23.4	23.1	−0.3	28.3
	5	37	24.4	25.8	1.4	32.9
	3	76	21.7	22.0	0.3	30.0
		Mean	23.2	23.6	0.5	30.4
Butyrate	3	10	26.5	27.8	1.3	31.1
	2	37	21.3	25.2	3.9	33.6
	6	77	18.9	21.1	2.2	27.4
		Mean	22.2	24.7	2.5	30.7

[a] Per cent of CO_2 derived from infused plus endogenous acid =

$$\frac{\text{Specific activity of expired CO}}{\text{Specific activity of acid in blood}} \times \frac{\text{Number of C atoms in infused acid}}{\text{Number of C atoms in expired CO}} \times 100.$$

[b] Per cent of energy requirement for maintenance derived from infused acid =

$$\frac{\text{Specific activity of expired CO}}{\text{Specific activity of infused acid}} \times \frac{\text{Moles of expired CO}}{\text{Kilocalorie required for maintenance}} \times \frac{\text{Kilocalorie per mole of infused acid}}{\text{Number of C atoms in expired CO}} \times 100.$$

to represent the maximum rate at which the infused acids were oxidized in this experiment. In addition, the per cent of expired CO_2 derived from infused plus endogenous acid was calculated (6). As expected, these values (Table 3) were slightly higher than those based on infused acid alone. The per cent of CO_2 derived from endogenous acid alone was obtained subsequently by difference. These values (Table 3) indicate that endogenous acetate made the greatest contribution to expired CO_2. Butyrate was intermediate, whereas propionate made little or no contribution. These observations are in accord with the suggestion made previously relative to differences in size of the body pool of each of these acids. The data on expired CO_2 were used also to compute estimates of the per cent of maintenance energy derived from the metabolism of infused acid. These values, also presented in Table 3, were somewhat higher than the corresponding ones for per cent of CO_2 derived from infused acid. Thus, they further substantiate the conclusion stated previously that the young milk-fed calf is capable of metabolizing appreciable quantities of these acids.

The total losses of C^{14} during the 11-hr collection period, both in urine and as expired $C^{14}O_2$, were relatively uniform (Table 4). The magnitude of these losses did not seem to be affected greatly either by the age of the calf or the kind of acid infused. In general, the results indicate that approximately two-thirds of the total dose of infused acid was oxidized to CO_2 during the 11-hr period.

METABOLISM OF INFUSED ACIDS

The chromatographic analyses of blood usually revealed detectable quantities of at least 11 organic acids. These were C-5 and longer fatty acids, butyric, propionic, acetic, formic, β-hydroxybutyric, succinic, lactic, malonic, glycolic, oxalic, malic, citric, and isocitric.

Since this study involved the use of calves at different stages of postnatal development, the data from individual infusions were ex-

TABLE 4

Total losses during 11-hr collection period of C^{14} in the urine and as expired CO_2

Infused substance	Calf No.	Calf Age	CO_2	Urine	CO_2 + urine
		(days)		*(% of total C^{14} infused)*	
Acetate	1	10	75	7	82
	4	10	62	5	67
	5	10	72	1	73
	3	37	66	4	70
	2	80	68	5	73
		Mean	69	4	73
Propionate	2	10	62	7	69
	5	37	66	6	72
	3	76	68	7	75
		Mean	65	7	72
Butyrate	3	10	63	9	72
	2	37	69	10	79
	6	77	66	7	73
		Mean	66	9	75

amined carefully to ascertain the effect of age on the metabolism of infused acids. There appeared to be no marked effect of age on either the concentration of organic acids in blood or in their degree of C^{14}-labeling. This is in accord with the previous observation that age had little or no effect on either the per cent of infused acid oxidized to CO_2 or the contribution of infused acid to the maintenance requirement for energy.

In the absence of an age effect, therefore, the results are summarized in Table 5 according to the acid infused. The acids included in this table are those present in concentrations greater than 10 μmoles per 100 ml of blood, or which changed either in concentration or specific activity during the infusions. Since all of the acids were labeled by the end of the first hour, the metabolic pathways involved must be discussed and evaluated in terms of changes both in concentrations of specific acids and in their degree of C^{14}-labeling. Comparisons of C^{14}-labeling in acids from calves of different ages required the calculation of relative specific activity values, since the total quantity of infused acid varied according to the weight of the calf, whereas the quantity of C^{14} was held constant, 400 μc.

Metabolism of acetate. The infusion of acetate increased the blood levels of acetate, β-hydroxybutyrate, lactate, and malate, whereas concentrations of other acids did not change appreciably. When the infusion was terminated, the level of acetate in blood decreased to the preinfusion level by the 11th hour. Except for acetate, the greatest increase in relative specific activity was exhibited by β-hydroxybutyrate and malate.

Increases in both concentration and relative specific activity of β-hydroxybutyrate indicate that acetate is quite active in ketone-body formation. It should be emphasized, however, that long-term infusions of labeled compounds make it difficult to assess whether labeled endogenous compounds arise directly or indirectly from infused material, except when the specific activity of the product approaches that of the infused compound.

The presence of labeling in fatty acids containing more than two carbons, particularly butyrate, suggests that acetate was being utilized to some extent for lipid synthesis. Most of the infused acetate was likely being metabolized via acetyl-CoA and the citric acid cycle, since a large percentage of the infused C^{14} was expired as $C^{14}O_2$.

Blood levels of acetate increased much more than was reported by Davis et al. (7), who infused acetate intravenously into a mature steer. The highest level of acetate infused by these workers would have provided about half of the maintenance energy requirement, as in the present work, yet blood acetate levels increased only from two- to tenfold. In the present study, blood levels of acetate increased from 10- to 50-fold, with an average increase of about 25-fold. In the present study, acetate concentrations continued to increase until the infusion was completed, whereas Davis and his co-workers observed fairly constant levels after an initial increase during the first hour of infusion. Reasons for these differences

TABLE 5

Mean concentrations and relative specific activities of organic acids in blood of calves subjected to acetate, propionate, or butyrate infusions

Acid in blood	Mean concentration (by hours)					Mean relative specific activity [a] (by hours)			
	0	1	4	8	11	1	4	8	11
	(μM per 100 ml of blood)								
Acetate infusions [b]									
Butyric	1.9	1.8	0.8	1.5	1.3	6.0	6.5	17.3	8.4
Propionic	2.2	2.0	1.7	2.1	2.1	5.0	6.8	12.2	5.7
Acetic	21.1	175.0	394.0	514.0	29.9	44.5	91.9	88.4	9.4
Formic	51.8	52.8	43.5	36.4	43.6	4.4	6.7	21.6	1.7
β-Hydroxybutyric	6.0	6.6	9.1	22.7	8.4	29.0	30.0	43.5	6.4
Lactic	156.0	306.0	298.0	284.0	276.0	1.4	5.6	9.3	4.3
Glycolic	17.4	18.6	17.2	14.7	15.2	2.9	8.4	16.4	4.9
Malic	4.8	5.9	7.6	11.1	8.7	4.3	21.2	44.3	17.8
Propionate infusions [c]									
Butyric	0.8	0.5	0.5	0.6	0.7	8.6	9.1	3.5	2.2
Propionic	1.5	246.0	214.0	353.0	1.6	98.9	97.7	98.8	22.8
Acetic	17.8	32.4	26.8	29.1	23.4	5.7	8.2	9.0	0.6
Formic	45.9	35.8	22.1	19.8	39.4	4.7	7.7	6.7	0.8
β-Hydroxybutyric	7.4	4.7	4.8	5.2	6.0	11.6	7.0	7.0	2.0
Lactic	132.0	288.0	411.0	422.0	295.0	5.7	10.7	14.3	3.1
Glycolic	15.9	13.2	8.0	7.0	14.8	4.6	12.1	14.8	2.0
Malic	3.9	4.5	3.6	3.2	6.0	8.5	21.1	36.2	9.3
Butyrate infusions [c]									
Butyric	0.6	18.6	24.4	40.1	1.8	68.8	94.8	93.0	8.5
Propionic	1.6	2.0	2.5	2.7	2.9	25.7	28.5	24.8	5.1
Acetic	17.1	22.5	21.9	29.6	27.3	1.5	2.5	2.5	0.6
Formic	31.5	29.1	31.0	30.0	32.1	3.7	7.0	9.9	0.9
β-Hydroxybutyric	7.3	17.0	15.4	12.8	6.4	33.4	32.0	32.7	2.6
Lactic	143.0	235.0	173.0	377.0	298.0	1.7	5.4	5.1	1.1
Glycolic	11.5	12.0	9.7	10.4	12.8	3.0	6.7	9.1	2.6
Malic	3.0	2.9	2.8	8.7	10.2	6.4	21.4	29.9	10.4

[a] Relative specific activity = $\frac{\text{Specific activity of acid in blood}}{\text{Specific activity of infused acid}} \times 100$.

[b] Average of five infusions.

[c] Average of three infusions.

are uncertain. Possibly, the maximum capacity for acetate oxidation was reached, whereas Davis and his group stated that the maximum rate of acetate oxidation was not reached with the mature steer.

Metabolism of propionate. Propionate infusions increased the concentration of propionate, acetate, and lactate in blood, but lowered that of formate, glycolate, and possibly β-hydroxybutyrate and malate.

The relative specific activity values show that propionate was very constant during the infusions; malate increased markedly by the eighth hour, lactate was higher and β-hydroxybutyrate was lower than during the infusion of either acetate or butyrate. The low relative specific activity of β-hydroxybutyrate indicates that significant amounts of ketone-bodies are not being formed directly from propionate.

The fixation of CO_2 to propionate, forming succinate, probably is the major pathway of propionate metabolism, yet there is little evidence in the present study whereby this can be evaluated in the milk-fed calf. The concentrations of succinate and other citric acid cycle intermediates were too low to assess the meaning of changes in specific activity. Nevertheless, the fact that a large percentage of the infused propionate can be accounted for in expired CO_2 leaves no doubt that this acid can be oxidized readily by young calves.

The relative specific activity values for lactate are slightly greater after propionate infusions than after either acetate or butyrate infusions. The over-all average relative specific activities (mean of 1, 4, and 8 hr) of lactate for infusions of acetate, propionate, and butyrate were 5.4, 9.9, and 4.1, respectively. Corresponding concentrations of lactate were 296, 374, and 262 μmoles per 100 ml of blood. Thus, both relative specific activity and absolute con-

centration values for lactate are greatest when propionate is infused.

Another effect of propionate infusion was the decrease in concentration of several organic acids in blood, such as formate, glycolate, β-hydroxybutyrate, and malate. The factors involved in this depression are unknown except that β-hydroxybutyrate was probably depressed by the glycogenic effect of propionate. In contrast, both acetate and butyrate infusions increased the concentration of β-hydroxybutyrate, glycolate, and malate.

Metabolism of butyrate. During butyrate infusions, increased concentrations of butyrate, acetate, β-hydroxybutyrate, lactate, and malate were observed. β-hydroxybutyrate and malate attained higher relative specific activities than lactate. The increased concentration of β-hydroxybutyrate and the high relative specific activity indicate that a portion of the butyric acid was oxidized to β-hydroxybutyrate. This reaction is in accord with known production of ketone bodies from butyrate. Though a high relative specific activity of propionate was observed, this is believed to be a result of contamination by tailing from the butyrate peak during chromatographic separation, particularly in view of the fact that there was not a marked increase in the concentration of propionic acid.

There is nothing in the results to suggest that butyrate was oxidized to CO_2 by other than established metabolic pathways. It is of interest to note that the specific activity of acetate remains very low during butyrate infusions; in fact, it was even lower than when propionate was infused. Butyrate is oxidized to form two moles of acetyl-CoA. Even if the turnover of acetate is rapid, preventing a build-up of acetate in blood, the specific activity of acetate would still increase during infusion of labeled butyrate if acetyl-CoA arising from butyrate exchanges with the blood pool of acetate. Thus, it appears that acetyl-CoA formed from the first two carbons of butyrate is metabolized immediately and seems to exchange little with the acetate pool of the blood.

Effect of infusions on blood lactate. As stated previously, the infusion of either acetate, propionate, or butyrate was accompanied by an increase in concentration of blood lactate. It seems likely, however, that this phenomenon is not due entirely to the conversion of infused acid to lactate. First, increases in blood lactate were usually quite marked, even for acetate and butyrate infusions, yet there was only a moderate increase in the relative specific activity of lactate. Secondly, the 11-hr concentration of lactate, approximately 3 hr after cessation of the infusion, was still appreciably higher than the preinfusion level, even though the 11-hr concentration of acetate, propionate, or butyrate had returned to the preinfusion level.

Effect of infusions on blood glucose. The preinfusion levels of blood glucose decreased slightly as the calves became older. The mean values for 10, 37 and 80 days of age were 89, 72, and 67 mg per 100 ml, respectively. This trend is in accord with the observations of other investigators (14, 15, 19). In the present study, however, age did not seem to have an appreciable effect on the changes in blood glucose induced by fatty acid infusion. Thus, the results are summarized in Table 6 without regard to differences in age.

Infusion of all three acids resulted in a definite drop in blood glucose levels. Acetate resulted in the smallest depression, the effect of butyrate was intermediate, and propionate caused the most severe decrease. It is of interest to note that the infusion of acetate increased the level of blood glucose slightly during

TABLE 6

Mean changes in concentration and specific activity of glucose in blood from calves infused with acetate, propionate, and butyrate

Substance infused	Hour 0	1	4	8	11
		Concentration (mg per 100 ml)			
Acetate[a]	89.2	99.6	53.8	66.9	78.3
Propionate[b]	74.2	42.1	25.9	31.8	79.4
Butyrate[b]	65.3	51.1	33.7	40.1	109.5
		Specific activity (dpm per μM)			
Acetate		12.3	50.2	75.9	34.1
Propionate		42.5	187.2	256.6	78.0
Butyrate		43.6	154.3	236.6	31.5

[a] Average of five observations.
[b] Average of three observations.

the first hour. The increase in glucose concentrations at the eleventh hour presumably was a combined result of the discontinuance of infusion at the eighth hour and the feeding of milk to the calves at this time.

The reason for the marked depression of blood sugar by propionate is unknown. The infusion of this acid also resulted in decreased levels of formate, glycolate, and possibly β-hydroxybutyrate and malate. The decrease in blood glucose caused by butyrate is in contrast with the results of Kronfeld et al. (11), who infused sodium butyrate intravenously into mature sheep and observed an increased level of blood glucose. This discrepancy may be related either to the fact that Kronfeld and his group were using mature ruminants, whereas the subjects in the present study were young milk-fed calves, or that there were differences in the levels of infused butyrate and in the duration of infusions.

The specific activity values for glucose in Table 6 seem to indicate that propionate is the best precursor of glucose, acetate the poorest, and butyrate is intermediate. However, the rank of the acids with respect to blood glucose concentrations during the infusion was always the reverse of relative specific activity, which makes it difficult to ascertain which acid is the better precursor of glucose.

Effect of infusions on blood malate. The infusion of both acetate and butyrate resulted in noticeable increases in both the concentration and the relative specific activity of malate in the blood (Table 5). The infusion of propionate produced a similar increase in relative specific activity of malate, though it had little or no effect on the concentration of this acid. The labeling of malate by acetate, propionate, and butyrate undoubtedly is due, in part, to the oxidation of these acids via the tricarboxylic acid cycle.

If the glyoxylate shunt is operative in the young calf, this might explain the increased levels of malate in blood during the infusion of acetate and of butyrate. In this pathway, isocitric acid is cleaved to form succinic and glyoxylic acids, which reaction is followed by a combination of glyoxylic acid and acetyl-CoA by malate synthetase to form malic acid.

If the glyoxylate cycle were operative in the calf during these infusions, the specific activity of C_1 of malic acid, which arises from acetate, should have a higher specific activity than C_1 arising from glyoxylate (3). To explore this possibility, part of an 8-hr malic acid sample from an infusion of acetate in a ten-day-old calf was decarboxylated by the procedure of Benson and Calvin (1). The results showed a specific activity of five disintegrations per minute per micromole for the C_1-carbon and a specific activity of 32 for the C_4-carbon, further suggesting the presence of the glyoxylate pathway or, more specifically, the presence of malate synthetase.

DISCUSSION

The present work shows that young milk fed calves, not actively producing volatile fatty acids by bacterial fermentation in the rumen, have the metabolic machinery necessary to obtain a significant portion of their maintenance energy from the metabolism of individual volatile fatty acids. Previous results by Martin et al. (13) indicated that such acids are utilized by ruminants as young as 3 wk of age, but did not provide quantitative information on the levels metabolized.

Sutton et al. (21, 22) demonstrated that calves fed only milk have a very limited ability to absorb acetic acid from the rumen, whereas those fed hay and grain increased steadily in absorptive ability. A possible explanation offered for their results was that accumulation of acetate in the blood, resulting from an inability to metabolize acetic acid, limited absorption. Present results seem to exclude that possibility.

The sites at which volatile fatty acids are metabolized in the milk-fed calf are unknown, though it seems probable that liver and muscle tissues are major sites. Sutton et al. (23) showed that mucosa from the rumen of milk-fed calves did not metabolize acetate, propionate, or butyrate, either singly or in mixtures, as fast as did mucosa from calves fed hay and grain. Therefore, the rumen tissue probably made little or no contribution to the metabolism of volatile fatty acids in the present study.

In general, results of this study have indicated that acetic, propionic, and butyrate acids are metabolized in the milk-fed calf by pathways well-established in other mammals. Possible demonstration of the glyoxylate pathway for the net synthesis of carbohydrates from fat is unique, in that this pathway has not been reported for animals. Madsen (12) tested chick embryos and rats fed high-fat diets, both of which were suspected of converting fat to carbohydrate. In neither case could he find evidence for either the formation of malate or the presence of the necessary enzymes. A preparation from livers and kidneys of rats, guinea pigs, and rabbits was recently found to contain malate synthetase activity, but isocitritase activity could not be shown (8).

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SEPARATION AND QUANTITATIVE DETERMINATION OF FOUR ORGANIC ACIDS FROM WINE BY PARTITION CHROMATOGRAPHY[a]

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INTRODUCTION AND REVIEW OF LITERATURE

The variations of organic acids in wine, both as to kinds and amounts, are important factors in the determination of the quality of wine. There is abundant evidence that these variations cause both chemical and physical changes in wines thus playing an important role in the flavor, color and keeping quality of this beverage.

In wine analyses, most of the currently available methods for the determination of organic acids are restricted to individual acids and prove unsatisfactory when several acids are to be determined in the same sample. A review of the difficulties of determining the organic acids and a comparison of methods have been given by Saburov **et al.** (8). Amerine (1) has mentioned the difficulties encountered in the determinations of various acids in wines, including malic acid.

Partition chromatography has been used more recently in the successful separation of organic acids. Martin and Synge (6) described a new form of chromatogram, depending not on adsorption on a solid phase, but on partition of solutes between two liquid phases. Adsorbing water in silica gel was used as one phase of the chromatogram, the other being some fluid immiscible with water, the silica acting merely as a mechanical support. Isherwood (4) evolved a method based on an improved modification of the above partition chromatogram which quantitatively separates many of the acids commonly found in fruit.

In 1950, Marvel and Rands (5), reported a similar chromatographic method, suitable for separation of various organic acids, except they have systematically increased the polarity of the developing solvent. Increasing amounts of n-butyl alcohol to the n-butanol-chloroform eluant provides more polarity in the development of the more water soluble acids. As little as 0.5 mg of an acid can be detected in an 80 mg. mixture under proper conditions. This method, however, is not especially well adapted to biological materials as it fails to separate some of the commonly occurring natural acids.

Three reports appeared in 1952 concerning the separation and identification of metabolically important acids; Donalson **et al.** (3), Phares **et al.** (7) and Bulen **et al.** (2). The first report suggested a simple device which permits the delivery of the solvent automatically and gradually increasing the polarity. This method has limited usefulness, either because accurate identification was difficult or because the research workers failed to include many important naturally occurring acids. Phares group suggested the possibility of satisfactory separation of a number of organic acids by the successive use of two solvent systems--butanol in chloroform, followed by ethyl ether, with 0.5N sulfuric acid on celite as the stationary phase in both cases. The difficulties with this method involved the rechromatographing of acids, not resolved with butanol-chloroform, on ethyl ether columns. While the results were satisfactory, the organic acid recoveries from celite columns were somewhat lower than the values obtained with silica gel.

Bulen et al. (2) have successfully extended the method of Marvel and Rands (5) toward complete separation and accurate identification of 16 important metabolically occuring acids. Their procedure uses an initial separation on a silica gel column followed, when necessary, by additional separations using both chromatographic and chemical techniques. They applied their method to the acids in tomato fruits and **Bryophyllum** leaves.

In the present study, the authors have applied the preliminary survey techniques of Bulen et al. (2) to separate and identify quantitatively the most important organic acids from wine: acetic, malic, citric and tartaric. The results of the study of three kinds of New York State experimental wines, Niagara, Delaware and Catawba, are presented.

EXPERIMENTAL

The complete procedure for the isolation of all organic acids in the experimental wines was not undertaken. In general, the preliminary or survey separation proposed by Bulen and associates (2) was followed. The survey separation was conducted by adding first, known quantities of specific acids, and then mixtures of these organic acids to the top of a silica gel column and developing the chromatograms with a series of n-butyl alcohol-chloroform solvents. Appropriately treated aliquots of wine samples were subjected to the same survey separation in this study, resulting in separation of acetic, malic, citric and tartaric acids. Fractions of the effluent were collected and titrated to obtain the amounts of acids present in the samples.

Apparatus. This is best appreciated by reference to Figure 1.

Reagents

1. Mallinckrodt's **silic acid** (specially prepared for chromatographic analysis) is used, after removal of the fine particles through repeated suspensions in distilled water—at least 1/3 must be removed—in order to facilitate packing a uniform column and reduce the pressure required to obtain a satisfactory flow rate; it is filtered, dried in an oven at 100°C for 24 hours and stored in a closed container.
2. Eluting solvents: mixtures of chloroform, washed twice with distilled water and c.p. n-butanol, to contain 5, 15, 25, 35 and 50% (v/v) n-butanol in chloroform. Each solvent mixture is equilibrated against 0.5N sulfuric acid by shaking the two phases in a separatory funnel and passing the solvent layer through dry filter paper, to remove suspended water droplets.
3. Standard 0.5N sulfuric acid.
4. Standard 2.0N sulfuric acid.
5. Standard 0.01N sodium hydroxide (carbonate free).
6. Phenol red indicator.

Column Preparation. A glass-wool plug is placed in the bottom of the chromatographic tube to support the column. Eight grams of the prepared silica gel are mixed with 5.5 ml of 0.5N sulfuric acid in a mortar. The resulting free-flowing powder is slurried in 60 to 70 ml chloroform and added to the chromatographic tube in successive portions. A gas pressure of 5 to 10 cm of mercury is applied to the top of the tube, to speed the removal of excess solvent, care being exercised not to allow the solvent level to fall below the top of the column.

Column Standardization. The mixture of known free organic acids containing a total of 10 to 70 ml dissolved in 0.5 ml of 0.5N sulfuric acid is mixed thoroughly with 1 gram of dry silica gel and the resulting free-flowing powder is transferred quantitatively to the top of the column. This transfer is easily accomplished by

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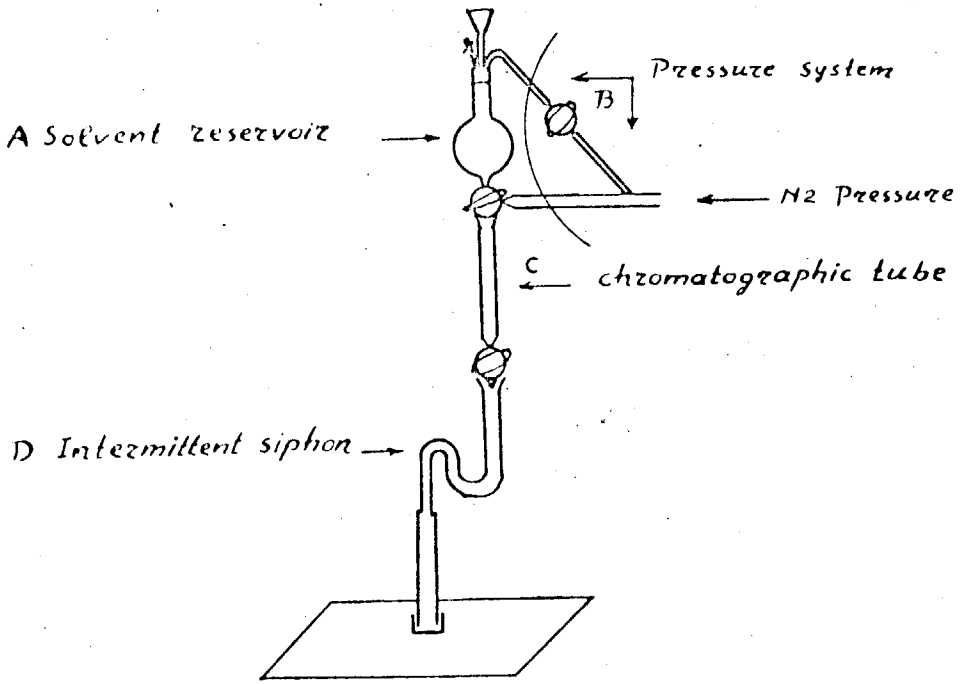

Figure 1. Chromatographic apparatus. The chromatographic tube is 2 mm in inside diameter, 24 cm long, with attached stopcock. The intermittent siphon delivers 3.5 ml.

pouring the mixture into the tube through a short-stemmed funnel which, along with the container is rinsed with 2 to 3 ml of chloroform.

Five ml of the 5% solvent are poured directly into the tube above the sample and the remainder is added to the reservoir, which permits the application of pressure from a nitrogen tank and at the same time allows the addition of more solvent to the reservoir without interrupting the pressure at the top of the column.

The n-butanol solvent schedule is as follows: 100 ml of 5%; 135 ml of 15%; 100 ml of 25%; 300 ml of 35%; 150 ml of 50%. Bulen et al. (2) reported that this schedule was experimentally selected to give maximum separation. The distribution coefficient determines the phase selected.

The gas pressure is adjusted so that the effluent is collected at the rate of one fraction (3.5 ml) per two and a half minutes. Rates greater than this tend to produce unsymmetrical curves and spreading of acid bands. Extremely slow rates are disadvantageous because of the increased time required and the increased losses due to esterification and diffusion. The effluent passes through an intermittent siphon adjusted arbitrarily to deliver about 3.5 ml. Individual fractions are collected in test tubes for titration in 50 ml Erlenmeyer flasks to which about 10 ml of carbon dioxide-free distilled water and 2 drops of phenol red indicator are added. The titrations are carried out using 0.01N sodium hydroxide from a microburet. Near the end point vigorous agitation is necessary to ensure intimate contact between the two phases. The complete preliminary separation requires about 9 hours.

Survey Separation Standardization. With these techniques pure acid mixtures were analyzed and the results are given in Figure 2 and Table I. The order of elution of the acids were determined by chromatographing the known acids singly, in pairs, in mixtures and all together. Besides the acetic, malic, citric and tartaric acids, mixtures containing succinic, pyruvic and formic acids for more accuracy of standardization were employed also.

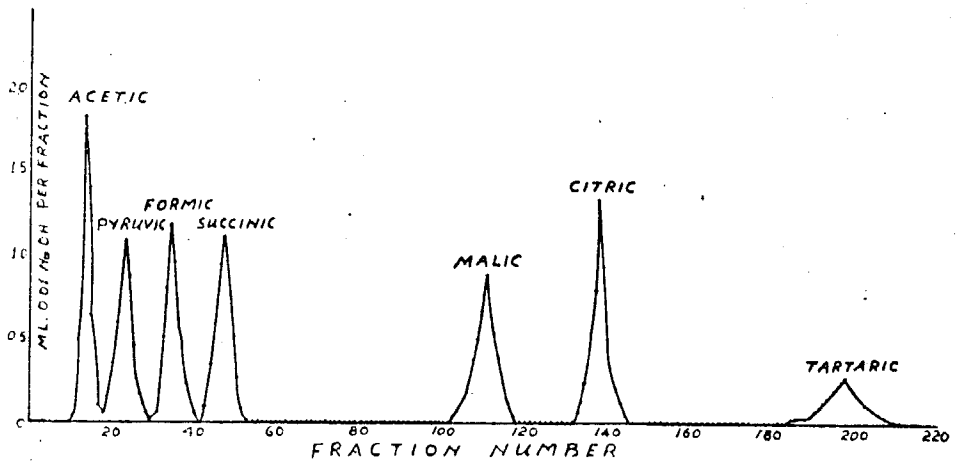

Figure 2. Results of column standardization.

Figure 2 was obtained by plotting each value of ml of 0.01N sodium hydroxide against the fraction number and shows the complete separation of acetic, malic, citric and tartaric acids during the preliminary or survey separation. Pyruvic, formic and succinic acids were separated also in this standardization of the column. The threshold volume of each acid is characteristic for the standardized column and permits the identification of the acids in question. The reproducible location of the mode of the curves at the same position on the abscissa under comparable experimental conditions demonstrates the specificity of this method.

Table I shows the excellent recovery of the organic acids from various mixtures. The poorest recovery ranged from 94.3 to 108.4%, which occurred in the case of formic acid; the average recovery, however, was 100.8%. The average recovery for the acids in this study were as follows: acetic, 99.5%; malic, 98.3%; citric, 100%; tartaric 102%. Bulen **et al.** report that the sensitivity of the method permits detection and tentative identification of quantities as small as 2 to 10 microequivalents of the individual acids.

Application For Wine Samples. Sample

TABLE I
Separation and Recovery of Known Mixtures of Acids

Expt. No.	Acetic	Pyruvic	Formic	Succinic	Malic	Citric	Tartaric
	Milligrams of Acid Recovered*						
1	10.31						
2	10.62		10.84				
3	9.90	9.90					
4	9.54	10.27	10.16				
5	10.07	9.58	9.43	10.30			
6						10.17	
7				10.30	10.10		
8	9.82	9.65	9.90	9.70	9.56	9.82	10.2

* 10 mg of each acid originally placed on the silica gel column.

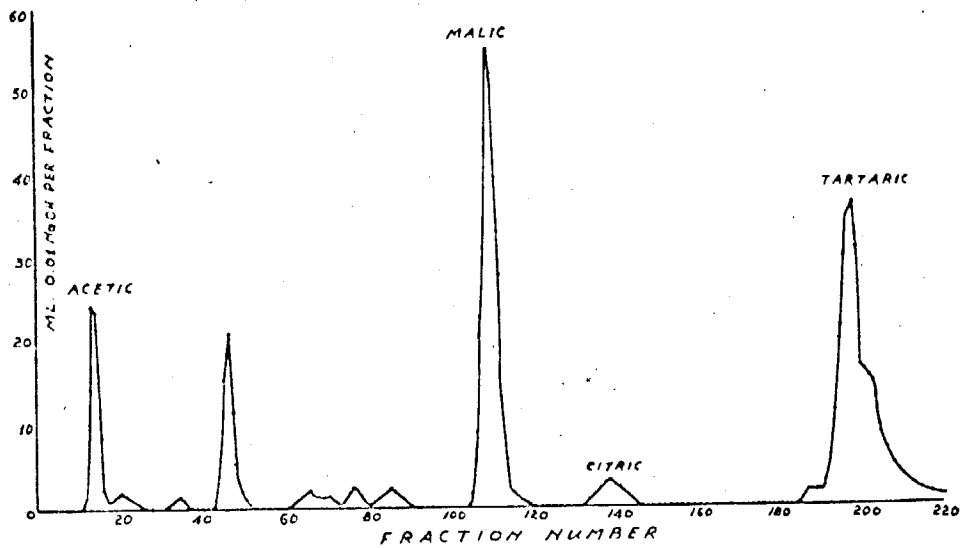

Figure 3. Results of preliminary separation of wine (variety Niagara).

Five ml of wine to be analysed are treated in the following manner before being placed on the column. The aqueous solution of the sodium salts of the organic acids, obtained after neutralization of the sample to pH=8.5 (at this pH the organic acids are completely neutralized) are evaporated in vacuo at room temperature. It is necessary to concentrate the dilute solution of the acids so that the separation of the acids on the chromatogram will be sharp. The addition of original aliquots of wine samples to the column give unsatisfactory separations.

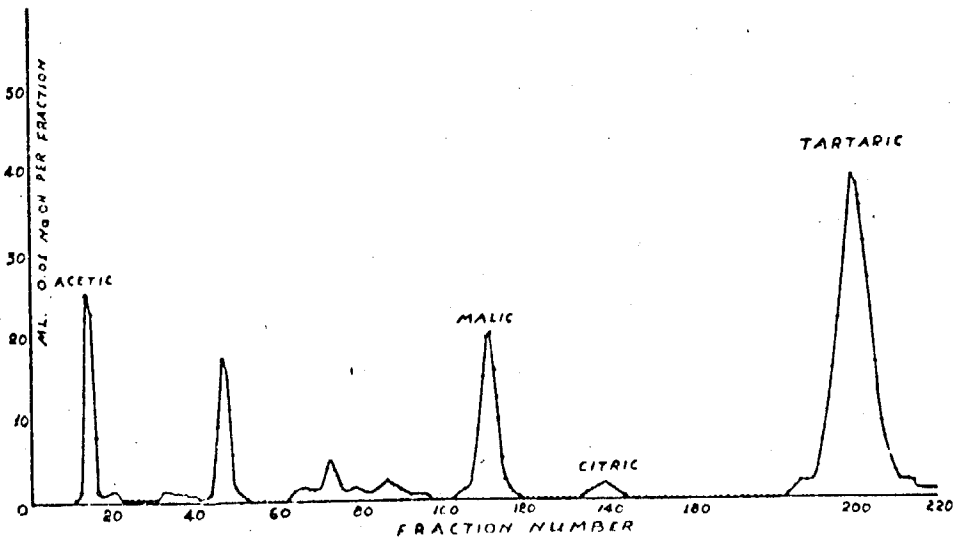

Figure 4. Results of preliminary separation of wine (variety Delaware).

The residue as a result of evaporation is acidified with the proper amount of 2N sulphuric acid to pH=2.0, to liberate all the acids from their salts; it is mixed thoroughly with about 1 g of dry silica gel and the resulting free-flowing powder is transferred quantitatively to the top of the column, as in the case of the mixture of known acids.

RESULTS AND DISCUSSIONS

Figures 3, 4, 5, show the separation of the acetic, malic, citric and tartaric acids from three kinds of wines made from Niagara, Delaware and Catawba grape varieties. They illustrate the complete separation of these acids by the survey procedure. The position of other acids, fraction numbers 20 to 100, can be observed in each experimental wine. These groups would require further separation for identification and quantitative determination.

The location of the mode of the curves is characteristically at the same position on the abscissa as in the case of the column survey standardization. The fraction at which each acid first appears and the fraction number making the complete release from the column are characteristic, indicating the identity of the isolated acids.

Table II lists the acids and the amounts found in these wines. As shown in this table, the organic acid content differs between each type of wine. However, no conclusions can be drawn about the factors responsible for the difference of organic acid content as this investigation was concerned only with the separation of the acetic, malic, citric and tartaric acids in the experimental wines.

It was of interest to note that citric acid was found to be at low uniform levels for these wine samples; and likewise acetic acid at approximately three times the level of citric acid. Tartaric acid was observed to be some twenty times greater than citric acid with a difference in the range of 0.2 g/100 ml of wine between the wine samples; the average content being 0.571 g/100 ml.

The greatest differences in acid content occurred with malic acid with a low

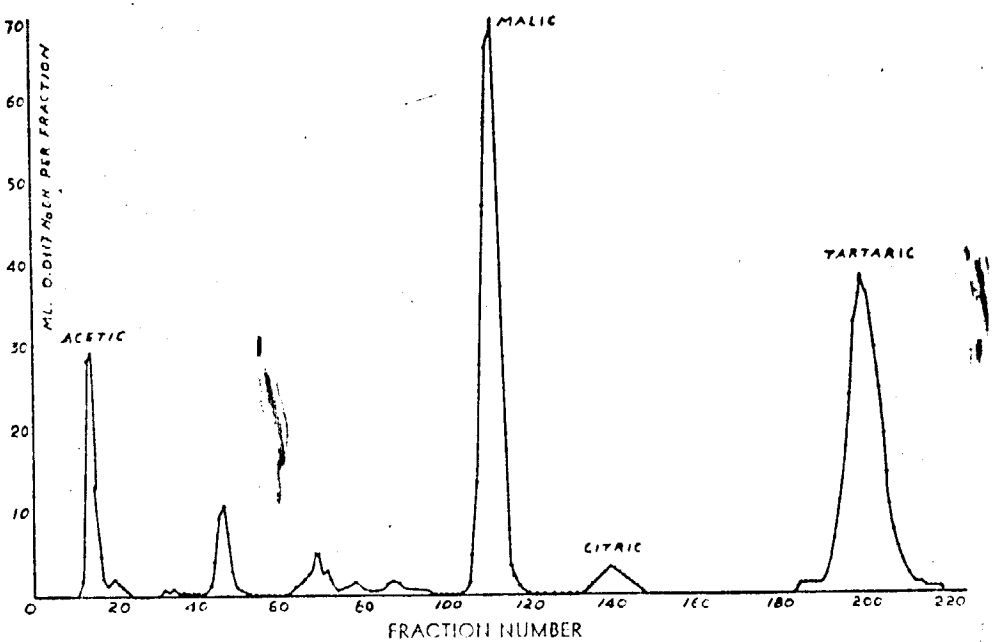

Figure 5. Results of preliminary separation of wine (variety Catawba).

TABLE II
Organic Acid Content of Experimental Wines

Wines	Acids, g/100ml of wine*			
	Acetic	Malic	Citric	Tartaric
Niagara	0.073	0.317	0.024	0.459
Delaware	0.073	0.141	0.016	0.591
Catawba	0.116	0.553	0.036	0.663

*Based on single determinations per sample.

of 0.141 g/100 ml in the Delaware sample and a high of 0.553 g/100 ml in the Catawba wine. It is to be remembered that these results are based only on a single determination per sample. Nevertheless, the recoveries obtained in the standardization and the sensitivity of the method indicates its reliability. It would thus seem that this survey procedure could be a very useful method for the separation and quantitative determination of malic acid in ripening grapes in warm regions, such as California. It could thus serve as a useful tool during the maturation and harvesting of grapes, as well as during the aging of the fermented product.

SUMMARY

A sensitive, reliable and satisfactory method for the separation and determination of acetic, malic, citric and tartaric acids in wines, by partition chromatography, is described.

After special treatment of the sample, the mixture of concentrated-free organic acids is separated using a partition chromatogram as has been described by Marvel and Randc and modified by Bulen, **et al.** The non-mobile phase consists of 0.5N sulphuric acid supported on a silica gel column, and the mobile phase of mixtures of n-butanol-chloroform. The polarity of the developing solvents is systematically increased. Each fraction obtained is titrated with standard sodium hydroxide and each value obtained is plotted against the fraction number.

The results of the separation and quantitative determination of acetic, malic, citric and tartaric acids from Niagara, Delaware and Catawba experimental wines are presented. It is suggested that this method might be useful for the quantitative determination of malic acid in ripening grapes.

ACKNOWLEDGMENT

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The Effect of Ethanol and Acetic Acid on Milk Yield and Milk Composition of Cows given Rations High in Concentrates

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It is an established fact that feeding cows high levels of concentrates results in a depression of milk fat under both field and experimental conditions. Several theories have been proposed as explanations (Van Soest, 1963). The foremost, advanced by Balch *et al.* (1952), is that diets low in hay and high in concentrates cause lowering of fat percentages by changing the relative proportions and, probably, the amounts of the volatile fatty acids (VFA) produced by fermentation in the reticulo-rumen. Since in the ruminants acetate is absorbed from the reticulo-rumen and has been shown by Popjak *et al.* 1951 (*a* and *b*) to be a source of a part of the milk fat, being built into saturated fatty acids containing up to 16 carbon atoms, a lowered production of acetate in the reticulo-rumen of cows receiving diets low in roughage and high in concentrates could be the cause of the fall in the fat content with these diets. Variable results have been obtained by feeding acetate salts (Mann & Shaw, 1947; Rook & Balch, 1961; Tyznik, 1951; Balch & Rowland, 1959) or acetic acid to cows with low milk fat. The second theory involves the reduction in the blood concentration of β-hydroxybutyric acid (Van Soest, 1963). Kumar *et al.* (1959) have demonstrated that mammary glands do utilize β hydroxybutyric acid, while the ketogenic activity of butyric acid and antiketogenic activity of propionic acid have been clearly demonstrated by Schultz *et al.* (1949). The third theory involves endocrine control of fat mobilization. Increased blood glucose levels suppress the liberation of free fatty acids (FFA) by adipose tissue, reducing the plasma lipids available for milk fat synthesis. The results obtained by Jorgensen *et al.* (1965) support the concenpt that the major factors depressing milk fat on high concentrate diets involve a high level of glycogenic metabolites that reduce blood ketones and lipid levels and tend to stimulate a fattening type of metabolism at the expense of milk fat synthesis. Ethanol when infused into the rumen (Ørskov *et al.*, 1967*a*) produces effects on the VFA content similar to those induced by acetic acid. It has been found that ethanol occurs in small quantities in the rumen in connection with overfeeding of starch (Allison *et al.*, 1964) and on purified diets for cows (Ørskov *et al.*, 1967*b*). Moomaw & Hungate (1963) have shown that pure cultures of some common ruminal bacteria produce ethanol, but that ethanol does not accumulate in the rumen. The explanation for ethanol formation in pure cultures may be that it is essential as a repository for hydrogen. Bornstein & Baker (1948) produced evidence to show that when the anaerobe *Clostridium kluyveri* was grown on ethanol with acetate present, it produced butyric and caproic acids. Butyric acid has been shown (Jorgensen *et al.*, 1965) to effectively relieve milk fat depression associated with high concentrates and caproic acid might be expected to give a similar effect since on degradation it yields C_2 units (Orskov *et al.*, 1967*b*). Ørskov *et al.* (1967*a*) subsequently showed that infusion of ethanol into the rumen of lactating cows gave rise to increases in milk fat percentages and in the proportions of acetic and caproic acids. As Nørgaard Pedersen (1967) pointed out that an appreciable part of silage consumed by dairy cows may consist of ethanol, it seemed appropriate to study the effect of feeding etha-

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Table 1. *Result of chemical analysis of the feedstuffs used in the experiments*

Period	Feedstuff	Dry matter (%)	Crude protein	Crude fat	Nitrogen-free extract	Crude fibre	True protein	Ash
			Percentage dry matter					
1	Clover-grass silage	23.92	21.91	7.07	35.08	27.09	13.92	8.90
2	Clover-grass silage	24.02	18.82	5.87	38.13	29.43	10.99	7.75
3	Clover-grass silage	24.68	13.49	6.04	39.79	29.21	7.09	11.47
All	Barley	85.77	10.49	1.94	80.56	4.47	9.89	2.55
All	Mixture 122	86.86	22.67	8.75	54.17	8.10	20.97	8.16
All	Mixture 121	91.53	41.61	11.43	31.26	8.04	39.53	7.81

Mixture 122 was a low-protein mixture consisting of 35% mixture 122, 31% rolled oats, 5% ground linseed, 21% ro d barley, 6% liquid molasses and 2% dicalcium phosphate.

Mixture 121 was a high-protein mixture composed of 44% cottonseed meal, 24% soyabean meal, 11% ground n meal, 8% sunflower meal, 7% liquid molasses and 2% dicalcium phosphate.

nol to lactating cows on their milk yield and composition and compare this with feeding them acetic acid. In addition, the effects of these two substances on the rumen volatile fatty acid composition and other metabolites in both blood and rumen contents were examined.

Experimental Procedure

Design and treatment

Three Danish Black and White milk cows (SDM) which had completed an average of 13 weeks' lactation, were used in the experiments designed to fit in with Latin Square model of three periods of 4 weeks. The first 3 weeks constituted a change-over period. Altogether three treatments were applied. Treatment A, which served as a control, consisted of a ration composed of silage, rolled barley and a concentrate mixture known as "Mixture 122"; treatment B, of silage, rolled barley, mixture 122 and another concentrate mixture called "Mixture 121", and ethanol. Treatment C was like B, but contained acetic acid in place of ethanol.

Composition of diet and level of feeding

Ration was adjusted according to milk yield of the individual cow during the first 2 weeks of change-over period and then stabilized during 3rd and 4th. The amounts of ethanol and acetic acid were calculated to supply approximately 20% of the total energy of the concentrates. On the average each cow received 659 and 1128 ml of ethanol and acetic acid, respectively. In compounding the experimental ration, allowance was made both for production and maintenance needs of the cows. Energy requirements were calculated according to the Danish standard norms, i.e. for maintenance, 1.5 × kg liveweight/200, and for milk yield, 0.4 × kg fat corrected milk (FCM), both expressed in Scandinavian Feed Units (SFU). Crude protein requirements were met by 0.7 g of digestible crude protein per kg body weight plus 65 g digestible crude protein per kg FCM.

The results of the chemical analysis of the feedstuffs are shown in Table 1. The silage contained 2.2% lactic, 0.9% acetic and 0.1% butyric acids together with 0.29% ammonia-N in dry matter and 0.4% alcohol (by weight). Its pH was 4.1. Mixture 122 was a high-protein type composed of 44% cottonseed meal, 24% soyabean meal, 11% groundnut meal, 8% sunflower meal, 7% liquid molasses and 2% diacalcium phosphate. Mixture 121 was low in protein and consisted of 35% of mixture 122, 31% rolled oats, 5% ground linseed, 21% rolled barley, 6% liquid molasses and 2% dicalcium phosphate.

Feeding

The cows were fed twice a day, at 0800 and 1600 hours. For a period of 2 weeks all the animals were acquainted with the standard ration. At the end of this period, ethanol and acetic acid were introduced into the diets according to the experimental design, particular attention being paid to gradually adapting them to their new rations

by adding small amounts of ethanol and acetic acid at the initial stages, and progressively increasing their quantity, such that, by the end of the change-over period, they were receiving the full determined amount. The amounts of ethanol and acetic acid allowed for the day were given in two equal portions. About a half of the amount of additive to be fed was mixed with 1 kg of concentrate and the other added to barley, which was thoroughly mixed with the concentrate, and immediately fed to the animals. There was no difficulty in getting the animals to eat the mixture containing ethanol, which they usually consumed within 10 minutes; but difficulties were encountered with acetic acid. When over 400 ml of it was given at a time acetic acid proved very unpalatable, probably because of its pungent smell, and it became necessary to dilute it with an equal volume of water before feeding. Occasionally, the cows could not consume all of the mixture even after dilution of acetic acid. The uneaten mixture was therefore weighed and the amount of acetic acid slightly reduced, while increasing the quantity of concentrates fed to compensate for the loss of energy which might otherwise have occurred. By the 3rd and 4th weeks, however, all the food allowed to the animals during each feeding period was eaten before the commencement of the next feeding. One exception to this occurred during the first period when there was some uneaten feed.

Collection of samples

Throughout the experimental period the animals were maintained in metabolic stands. For collection of urine they were fitted with urinary catheters. In one cow the catheter proved unsuitable and therefore it had to be harnessed with urinale. The cows were milked twice a day. During the collection periods the quantity of milk collected at each milking was weighted and a small sample taken. All the samples obtained at each milking for a period of 7 days were bulked and from these samples were taken for analysis of dry matter, fats and proteins. Faeces and urine excreted each day were weighed and stored in a deep freezer at −15°C. Urine was collected in 30 litre plastic bottles to each of which approximately 100 ml of 10% HCl had been added. On the 5th or 6th day during the collection period, samples of blood from the jugular vein and rumen liquor (taken by stomach tube) were obtained, usually 3 hours after the morning feeding. A small portion of the blood was collected into a test-tube and was used for urea determination (in serum), while the rest was kept in a 250 ml plastic bottle containing a few drops of heparin. Rumen liquor was collected with plastic flasks containing a few drops of toluene. All samples to be stored were covered by a 1 cm layer of paraffin and immediately deep frozen at −15°C.

Chemical analysis

Rumen samples were analysed for pH, volatile fatty acids, ethanol and ammonia, while in blood, alkaline reserve, urea, ammonia and ethanol were determined. The ethanol and nitrogen concentration of urine were also analysed. pH of rumen samples was immediately determined using glass electrodes (Radiometer, Copenhagen), while the alkaline reserve was determined on fresh unheparinized blood by potentiometrically titrating 0.2 ml of it in 5 cc of *N*/100 HCl saturated with quinhydrone with *N*/100 NaOH, (Ochia, 1967). Total VFA of rumen sample were determined after steam-distillation titrimetrically using automatic titrator (TTTlc, Radiometer, Copenhagen) and individual acids estimated by gas liquid chromatography incorporating a flame ionization detector. The column was packed with 20% carbowax 20M and 2% phosphoric acid on 100–120 mesh celite (Perkin-Elmer Corporation, Norwalk, Connecticut, U.S.A., 1963), using nitrogen as carrier gas and a column temperature of 120°C.

Analysis of faeces was made using standard methods. Ethanol determination was by the Widmark method as described by Lundquist (1959). Ammonia and urea were analysed by the Conway micro-diffusion technique, total nitrogen by the Kjeldahl method and lactic acid after the method of Barker & Summerson (1941).

Statistical analysis was carried out according to the Latin Square model with all the data obtained.

Results

The average feed intake, milk yield and its composition are shown in Table 2. It can be seen that there was an increase in milk yield and its fat content with ethanol and acetic acid supplementation. No significant difference was found between cows with respect to milk yield or its contents. When the animals received the control ration (treatment A) 11.91 kg of FCM were obtained as compared with the 13.81 and 15.05 kg obtained in treatments B and C, respectively. Acetic acid supplementation gave apparently higher yield of FCM than ethanol, which is caus-

Table 2. *Effect on milk yield and composition of substituting part of the concentrate of the basal[a] ration with ethanol or acetic acid*

Treatment	Yield/day		Milk fat		Non-fatty solid (%)	Milk protein (%)	Feed intake/day	
	Milk, kg	FCM, kg	Content, %	Yield/day, g			Dry matter, kg	Additives (ml)
A	14.50	11.91	2.77	402	8.91	3.33	10.0	
B (ethanol)	14.68	13.81	3.43	504	8.61	3.05	8.5	659
C (acetic acid)	15.66	15.05	3.71	581	8.67	3.13	8.0	1128
S.E.	1.24	1.17	0.13	53	0.12	0.11		

[a] Basal ration composed of clovergrass silage, rolled barley and concentrates.

ed by an increased milk fat percentage ($P < 0.1$). There was no significant ($P > 0.1$) change in either the non-fatty solid component of milk or its protein content. However, during the 3rd period there was a noticeable drop in milk fat percentages, as shown by the following figures: Period 1: 3.83; period 2: 3.63; and period 3: 2.45%. A similar drop did not occur in other milk components.

Table 3 gives the digestibility coefficients of the different feed components. This shows that there was no adverse effect on the digestibility of organic matter, true protein (in treatment B), crude protein, crude fibre and crude fat by feeding ethanol or acetic acid. There was an apparent decreased efficiency of nitrogen retention and balance as seen in treatments B and C. The reduction in nitrogen retention (percentage total N in faeces and urine of total N in feeds) might have been influenced by the increased amount of nitrogen given off with milk.

The pH and concentration of ammonia and organic acids in the rumen content are given in Table 4. It can be seen that the pH and concentration of the organic acids, especially lactic acid, appear to vary inversely. The diets containing ethanol and acetic acid gave a higher yield of total volatile fatty acids than the control ration. In this regard the influence of acetic acid appeared more pronounced than that of ethanol. Concentration of lactic acid rose insignificantly from 0.13/mmols/100 ml in treatment A to 0.16 and 0.16 mmols/100 ml in B and C, respectively. In fact the high average figures for the three treatments are due to a very high lactic acid content of 0.42 mmol/100 ml in the 3rd period against only small amount in the two first periods. The highest rumen ammonia concentration was observed with the ethanol diet. This might be related to decreased nitrogen retention observed with feeding ethanol. There was a definite increase in the relative proportions of acetic acid to the total VFA, mean values for treatments B and C being 59.3 and 59.6, as compared to 51.2 for treatment A. As was to be expected, molar proportions of propionic varied inversely to those of acetic acid; the values fell from 27.7 in treatment A to 22.3 and 25.2, respectively, in B and C. The changes in the molar proportions of butyric acid were similar to those of propionic.

Table 3. *Effect of substituting part of the concentrate of the basal[a] ration with ethanol* (B) *or acetic acid* (C) *on the utilization of various food components*

Treatment	Digestibility, %						Nitrogen utilization (%)	Nitrogen balance (g/day)
	Organic matter	True protein	Crude protein	Crude fat	N.F. extract	Crude fibre		
A	72.7	71.9	72.8	68.3	79.2	41.9	32.0	18
B	74.9	74.9	75.1	73.6	80.8	48.8	28.7	13
C	73.6	71.8	73.1	77.4	78.8	50.5	24.5	12
S.E.	1.81	2.04	1.90	1.24	1.93	5.31	2.09	6.5

[a] Basal ration composed of clover-grass silage, rolled barley and concentrates.

Table 4. *Effect of feeding ethanol* (B) *and acetic acid* (C) *on the pH and the concentration of NH_3-N, lactic acid and VFA in the rumen contents*

Treatment	pH	NH_3-N (mg%)	Lactic acid (mmols/100 ml)	VFA	Molar % of total VFA				
					Acetic	Propionic	Butyric	Valeric	Iso-Valeric
A	6.72	11.6	0.13	9.5	52.2	27.7	15.2	4.1	1.5
B	6.52	16.2	0.16	10.8	59.3	22.3	13.8	3.4	1.2
C	6.57	13.1	0.16	11.7	59.6	25.2	11.8	2.2	1.3
Period									
1	6.45	14.1	0.03	10.9	63.9	16.3	16.1	1.9	1.9
2	7.07	14.6	0.01	8.5	52.4	30.8	12.3	3.4	1.0
3	6.28	12.1	0.42	12.5	53.9	28.0	12.6	4.4	1.1
S.E.	0.21	1.90	0.009	0.21	7.16	5.79	1.97	0.42	0.39

In period 1 the proportion of acetic and butyric acids were higher and that of propionic lower than in the following periods. The proportions of acids higher than butyric, calculated as valeric and iso-valeric, appeared to decrease in treatments B and C, although the fluctuations in the proportions of iso-valeric were of very little significance.

Table 5 shows the changes in blood alkaline reserve, concentration of ammonia, urea and ethanol in rumen liquor and urine. There was an apparent rise in the alkaline reserve of blood in treatments B and C, with a greater increase in the latter. The concentration of blood ammonia nitrogen slightly decreased in treatment B, but displayed no change in treatment C. Urea concentration, on the other hand, obviously increased from 26.2 in treatment A to 30.1 mg% in ethanol treatment. However, there was a decrease with treatment C. It is interesting to note that with the ethanol treatment the concentration of NH_3-N tended to increase in the rumen contents, while in the blood it appeared to decrease, as compared with the control treatment. Blood ammonia concentration depends, among other factors, on the amount of ammonia being evolved in the reticulo-rumen (Ochia, 1967) and also on protein metabolism both in the liver tissues and body cells. The epithelial cells of the rumen mucosa have been shown to synthesize urea from ammonia (Aliev & Kosharov, 1966; Kosharov, 1963). This might lead to a lowered ammonia and an elevated urea concentration in the blood in the face of increasing ammonia concentration in the rumen content.

Blood ethanol concentration rose noticeably during ethanol and acetic acid treatment. Thus, in the control treatment it was 33 mg%, while in ethanol treatment it was 74 mg%, and in treatment C, 71 mg%. In the rumen content, however, there was not appreciable change in ethanol concentration. This might be due to the fact that ethanol does not accumulate appreciably in the rumen, being either quickly utilized there or absorbed through the rumen walls into the blood. In urine there was no noticeable change in ethanol concentration, but some amount of ethanol was excreted with urine in all the treatments.

Table 5. *Changes in blood alkaline reserve, concentration of ammonia and urea in blood, and ethanol in rumen liquor, blood and urine*

Treatment	Analysis of blood, mg%			Ethanol, mg%		
	Alkaline reserve	NH_3-N	Urea	Blood	Rumen content	Urine
A	151	5.6	26.2	33.4	3.3	5.6
B	173	4.2	30.1	74.2	3.5	4.5
C	198	5.5	23.7	71.3	4.6	5.0
S.E.	33.0	1.69	3.02	16.4	0.75	0.74

Disussion

The lower milk fat content which has been associated with rations high in concentrate and low in hay (Balch *et al.*, 1952; Balch *et al.*, 1955; Jorgensen *et al.*, 1965) occurred in our experiment as well. This might be related to the rise in rumen lactic acid content and molar proportion of propionic acid while that of acetic acid was lowered as the experiment proceeds. Balch *et al.* (1967) obtained a slightly increased milk yield and reduced milk fat content by adding to the diet of cows calcium salts of acetic, propionic and butyric acids. These findings contrasted from their earlier results obtained with the free acids (Balch & Rowland, 1959). They concluded that the specific effects of the acids were masked by a more marked general effect arising from their addition to the diet as calcium salts. Our findings confirm that adding free acetic acid to diet, concomitant to decreased milk fat content, increases the amount and quality of milk produced. Considerable energy is required by the mammary glands for the formation and secretion of milk. It has been shown that in the ruminant such energy can be obtained by the oxidation of acetate (Balch *et al.*, 1955). The acetate from which the udder synthesizes fatty acids may be obtained exogenously from the rumen via the blood (McClymont, 1949) and endogenously from the breakdown of higher fatty acids absorbed from the blood (Popjak, 1951*b*). The results of our experiments do show that the molar proportions of acetic acid of the rumen VFA increase noticeably when ethanol and acetic acid are included in the diets. Apart from milk fat synthesis, both acetate and propionate are important sources of energy for the body (Balch *et al.*, 1955). In this regard, therefore, it is interesting to note that the administration of acetic acid and ethanol caused an improvement in milk yield. This agrees with the early work of Rook & Balch (1961) who showed that acetic acid increased milk yield when infused intraruminally.

The effect of ethanol feeding on the proportions of VFA in the rumen content suggests that in the rumen the type of reaction, such as observed by Bornstein & Baker (1948) with *Clostridium kluyveri* grown on ethanol, might occur. Ørskov *et al.* (1967*a*) presume that ethanol in this reaction acts as the electron donor and source of acetyl CoA in the synthesis of butyric, valeric and caproic acids. No increase was obtained in the levels of butyric, valeric and iso-valeric acids in our experiments. Since it has been demonstrated by Moomaw & Hungate (1963) that ethanol is metabolized very slowly when added to rumen contents *in vitro*, it might be assumed that *in vivo* a greater part of the ethanol added into the rumen content is absorbed into the blood within the first 1–3 hours after administration. However, the amount metabolized in the rumen might have been sufficient to account for the observed increment in the acetic acid component. The fate of the other portion of ethanol absorbed through the rumen mucosa into the blood could not be accounted for from our experiments. But it is usually recognised that the liver is the main principal organ involved in the metabolism of alcohol (Pawan, 1968). Alcohol is known to be first converted to acetaldehyde, a reaction catalysed by the zinc-containing enzyme, alcohol dehydrogenase with the co-factor nicotinamide adenine dinucleotide (NAD) as the hydrogen acceptor (Lundquist *et al.*, 1959; Westheimer *et al.*, 1951). The acetaldehyde formed is converted to acetyl-CoA which then goes into the Krebs cycle and probably participates in other reactions. The former pathway is of particular importance, since it may involve the synthesis of fatty acids, cholesterol and ketone bodies. Riis *et al.*, (1960) have calculated that 50% of the fatty acids of milk fat could arise from blood lipids. The increase in milk fat content obtained by feeding ethanol might be partly accounted for by increased production of fatty acids in the liver. The feed value of ethanol may rely more on this metabolic pathway than on increasing the acetate level in the reticulo-rumen. On the other hand, Ørskov and his co-workers (1967*b*) found that rumen concentration of ethanol was high during the initial stages of ethanol infusion, but that as this progressed, the values diminished. Thus, according to the authors, some degree of adaptation by the rumen micro-organisms to coping with the ethanol was taking place. In their view a much greater portion of the infused ethanol was utilized in the rumen than was absorbed into the blood. It seems therefore that more work with, perhaps, radioactive ethanol is necessary to determine how much of the ethanol infused into or eaten by the cow is metabolized in the rumen or absorbed into the blood.

The effect on milk fat percentage is in agreement with that which might be expected from changes in VFA composition of the rumen contents and confirm the results of Balch *et al.* (1967, 1959) and Ørskov *et al.* (1967*a*). Butyric as well as acetic acid, is known to increase milk

fat percentage; but as mentioned above, there were no increases in either the butyric, valeric or iso-valeric acid. Analysis of caproic acid was not carried out in our experiments, but it has been assumed (Ørskov *et al.*, 1967*a*) that caproic acid acts in a manner similar to butyric acid as a precursor of milk fat. The level of caproic acid in the rumen has been demonstrated to rise 1 to 3 hours after feeding (Ørskov *et al.*, 1967*b*).

Blood alkaline reserve might be expected to fluctuate, within narrow limits, inversely with the amount of fatty acids being produced and absorbed from the rumen (Ochia, 1967). Furthermore, the fatty acids and ketone bodies, possibly β-hydroxybutyric acid (Van Soest, 1963), which might have been formed as a result of hepatic breakdown of ethanol, may weigh heavily on the buffering capacity of the blood.

Our results seem to be at variance with this view, since blood alkaline reserve apparently rose with the rumen VFA.

Since account was not taken of ethanol or acetic acid in calculating total organic matter consumed by the cows, the apparent increases in the digestibility of organic matter, true and crude protein, crude fibre and crude fat seem to suggest that the feeding of ethanol and acetic acid to cows on a ration high in concentrate does not adversely affect the efficiency of feed utilization.

Summary

1. An investigation was carried out on the effect of feeding ethanol and acetic acid on milk yield and its fat content and feed utilization by 3 cows on a diet consisting of concentrates, rolled barley and silage.

2. Milk yield per day increased from 11.91, on the basal ration, to 13.81 and 15.05 kg fat corrected milk with ethanol and acetic acid supplementation, respectively, and its fat content, from 2.77 to 3.43 and 3.71%.

3. The molar proportions of acetic acid of the total rumen VFA rose, while those of propionic, butyric and valeric acids decreased with ethanol and acetic acid feeding.

4. There were no adverse effects on the digestibility of organic matter, true and crude protein, crude fibre and crude fat with ethanol and acetic acid supplementation.

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Nutrition

BIOCHEMICAL EFFECTS IN YOUNG RATS FED EXCESS L-GLUTAMIC ACID. J. S. Adkins, L. M. Lewis* and J. F. Lynch, Jr.*. Division of Nutrition, FDA, Washington, D. C. 20204.

Glutamic acid (GA) is a widely used source of nonessential nitrogen and a salt substitute. The salts of GA are used as a flavor enhancer. GA is considered to be one of the least toxic amino acids. To better understand the biochemical effects of excess GA, Holtzman male rats (150g) were fed a laboratory chow diet with 50% added GA for 4 weeks. The rats maintained their weight and no deaths resulted. Weanling rats (46g) were fed laboratory chow supplemented with 25% GA for 5 weeks. L-arginine at 2% or pyridoxine (B_6) at 20ppm were added to the total diets with and without GA. Glutamic acid depressed growth 45% and reduced food efficiency (FE) 40%. Arginine partially restored growth and FE; B_6 had no effect. The kidneys and testes of rats fed GA were hypertrophied. No differences in liver and spleen weights were noted. Rats fed GA had a slightly lower glucose level (136 vs 159 mg %) and an elevated blood urea nitrogen level (18.1 vs 22.5 mg %). There was no difference in plasma cholesterol, total protein or other blood components measured. The results indicate that a large dietary excess of glutamic acid causes a growth depression that can be partially alleviated with L-arginine.

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Nutrition

EFFECT OF MONOSODIUM L-GLUTAMATE ON DEVELOPMENT AND REPRODUCTION IN RATS. J. F. Lynch, Jr.*, L. M. Lewis*, E. L. Hove and J. S. Adkins. Division of Nutrition, FDA, Washington, D. C. 20204.

Recent reports have indicated adverse effects of monosodium glutamate (MSG) in experimental animals [Science 164:719 (1969); Science 166:386 (1969)]. Holtzman virgin female rats (220g) were fed a stock maternity diet or the same diet supplemented with 10% MSG or 10% glutamic acid (GA) for 2 or 3 weeks prior to mating and throughout gestation and lactation. Weight gain (30g) before mating was the same for controls and treated rats. The rats reproduced normally and no differences in litter size (avg 10-12 pups) or birth weights (avg 6.5g) were noted. Gross observations and biochemical studies of selected blood components were made in neonatal, weanling and adult rats. Weanling rats from control or MSG-fed mothers grew the same when fed either a laboratory chow diet or the diet with 10% added MSG. Sodium acetate was added to one diet as a sodium control. In one study, 22 newborn rats were intubated daily from day 5-10 with a high dose of MSG (5g/kg body wt). After 8 weeks, there was a growth depression (42%) and increased vascularization of the iris. The results indicate that rats fed 10% MSG grow and reproduce satisfactorily but that growth of newborn rats is depressed by massive oral doses of monosodium glutamate.